THE INFLUENCE OF RELEASE MODALITY ON SYNAPTIC TRANSMISSION AT A DEVELOPING CENTRAL SYNAPSE

by

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A thesis submitted in conformity with the requirements for the degree of Ph.D.

Graduate Department of Physiology

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ABSTRACT

The auditory brainstem is comprised of a number of synapses specialized for the transmission of high-fidelity synaptic signals. Within the first three postnatal weeks, these pathways acquire the ability to process high-frequency signals without compromising timing information. However, little is known regarding developmental adaptations which confer this ability. Situated in the sound localization pathway, the calyx of Held-medial nucleus of the trapezoid body synapse provides an ideal model for investigating such adaptations as both the pre- and postsynaptic neurons are accessible to electrophysiological experimentation. Using this synapse, we have shown herein that the spatial coupling between voltage-gated calcium channels (VGCCs) and synaptic vesicles (SVs) tightens during development. Immature synapses use a loosely-coupled arrangement of many N- and P/Q-type VGCCs ("microdomain" modality) while mature synapses use a tightly-coupled arrangement of fewer P/Q-type VGCCs, to release SVs ("nanodomain" modality). As a consequence of this tightening, synaptic delay (SD) shortens. By fluorescence- and electron microscopy of SVs near active zones, we further identified the filamentous protein septin 5 as a molecular substrate, differentiating the two release modalities, which may act as a spatial barrier separating VGCCs and SVs in immature synapses. Finally, we have demonstrated that changes in release modality affect the nature of short-term plasticity observed at this synapse. Using trains of action potentials as presynaptic voltage-commands, we showed that, downstream of calcium influx, the microdomain modality promotes short-term facilitation in excitatory postsynaptic currents (I_{EPSC}), and calcium-dependent decreases in SD, with these being absent in synapses employing the nanodomain modality. In contrast, we found that as a result of depletion of SVs, short-term depression of I_{EPSC} dominates in synapses using the nanodomain modality, and correlates with calcium-dependent increases in SD.

These findings imply that the type of release modality has a significant impact on the strength and timing of synaptic responses. The microdomain modality imparts greater dynamic range in timing and strength, but does so at the cost of efficiency and fidelity, while the nanodomain modality is a key accomplishment consolidating the high-fidelity abilities of this synapse.
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1. INTRODUCTION
1.1 THE AUDITORY BRAINSTEM CIRCUITRY

1.1.1 Anatomy.

The essential circuitry for low-level processing of azimuthal sound placement in rodents resides in the auditory brainstem. A minimal schematic of this circuitry is overlaid on top of a photograph of a brainstem slice typical of those used during the course of the experiments presented herein (Fig. 1.1). These components comprise the superior olivary complex (SOC) which computes sound source location in space based on binaural signals of varying intensity or frequency phase-shift (see section 1.1.3). The success of both types of processing depends highly on the maintenance of accurate action potential (AP) timing throughout the circuit.

The neurons that make up the SOC are characterized by certain biophysical and morphological properties that make them particularly well suited for the transmission of precise timing information. These characteristics include; the prevalence of large diameter axons (Carr, 1993), large cell bodies with fewer dendrites (Carr, 1993), large calyceal presynaptic nerve terminals with many release sites (Taschenberger et al., 2002; Sätzler et al., 2002), fast presynaptic voltage-gated K⁺ and Na⁺ channels (Perney & Kaczmarek, 1992; Perney et al., 1992; Dodson et al., 2002; Leão et al., 2005; Nakamura & Takahashi, 2007), and postsynaptic inhibitory and excitatory receptors with rapid gating kinetics (Isaacson & Walmsley, 1995; Smith et al., 2000; Joshi et al., 2004; Koike-Tani et al., 2005). In addition, many of the nuclei within the auditory brainstem appear to have their neurons arranged tonotopically (see Oertel, 1999).

Sound signals are initially carried as pressure waves through air or other media. Reception of sound pressure waves (SPW) takes place in the cochlea where they are transduced by the tonotopically arranged inner and outer hair cells (IHC/OHC). Movement of cilia on these mechanoreceptive cells results in a wave-like pattern of depolarization from spiral ganglion neurons which is transmitted through the auditory nerve to the ipsilateral anterior ventral cochlear nucleus (aVCN) (Nomoto et al., 1964). These terminals supply excitatory input to both the globular bushy cells (GBCs) and spherical bushy cells (SBCs), which are specialized for high- and low-frequency sounds within the aVCN, respectively (Smith et al., 1991; Smith et al., 1993). In addition, terminals contacting SBC neurons within the aVCN form specialized endings called the endbulbs of Held as their primary inputs, while GBC neurons receive more numerous and smaller excitatory inputs (Brawer & Morest, 1975; Tolbert & Morest, 1982).

aVCN neurons phase-lock their output to the periodic input frequency of the hair cells and are the first relay at which the maintenance of AP timing becomes crucial. SBCs transmit their phase-locked excitatory signal to the tonotopically-arranged neurons of the medial superior olive (MSO) which is believed to be the integrating centre for interaural timing differences (ITD) in the localization of low-frequency sounds (Grothe, 2003). GBCs project thick axons across the midline of the brainstem and end in the giant axo-somatic calyx of Held nerve terminal within the medial nucleus of the trapezoid body (MNTB). The GBCs exhibit sharp phase-locking to their multiple inputs and transduce this into rapid APs which trigger similarly well timed excitatory transmitter release, via the calyx of Held, onto the principal neurons of the MNTB (Morest, 1968; Smith & Rhode, 1987; Spirou et al., 1990).
Figure 1.1: The Superior Olivary Complex Sound Localization Circuitry.
Schematic of synaptic connections overlaid on a photograph of a brainstem slice typical of those used for the electrophysiological experiments performed herein. Stimulation of the hair cells (HC) leads to excitation of the ipsilateral spherical and globular bushy cells (SBC/GBC) within the anterior ventral cochlear nucleus (aVCN). GBCs extend projections across the midline where they form the excitatory calyx of Held nerve terminal onto principal cells of the medial nucleus of the trapezoid body (MNTB). GBCs also extend excitatory projections to the ipsilateral lateral superior olive (LSO) where they are integrated with inhibitory terminals originating from ipsilateral MNTB neurons. This forms the interaural level difference (ILD) sound localization circuitry. SBCs extend excitatory inputs to the ipsilateral medial superior olive (MSO) where they are integrated with excitatory input from the contralateral MSO and inhibition from the ipsilateral MNTB. This forms the interaural timing difference (ITD) sound localization circuitry. + and - within the diagram refer to excitatory and inhibitory connections respectively.
It is generally accepted that MNTB neurons receive input from only one calyx of Held, however some accounts of multiple calyces innervating a single MNTB principal cell have been documented (Smith et al., 1991; Bergsman et al., 2004). Output from MNTB neurons innervates both the ipsilateral (in reference to the MNTB) MSO and lateral superior olives (LSO) where it converts excitation from the GBCs to inhibition. Transmission from the MNTB is primarily glycinegic with a smaller component mediated by GABA<sub>A</sub> receptors (Spangler et al., 1985; Smith et al., 2000). In both the LSO and MSO, the inhibitory drive from the MNTB is met coincidentally by excitation arriving from the ipsilateral (relative to the LSO/MSO) aVCN. It is the comparison of these inputs at both the LSO and MSO that is transmitted for higher processing in the inferior colliculus (IC) and dorsal nucleus of the lateral lemniscus (DLL) encoding interaural level differences (ILD) and ITD information respectively (Boudreau & Tsuchitani, 1968; Cant & Casseday, 1986; Glendenning et al., 1992; Grothe & Sanes, 1994).

1.1.2 The Calyx of Held-MNTB Synapse & its Role Within the Auditory Circuitry.

The calyx of Held-MNTB synapse acts as a sign-inverting relay within the circuitry converting GBC excitation to inhibition at the LSO. In addition, MNTB neurons receive more traditional bouton-style input which can be either excitatory or inhibitory, but the origins of which are not well known (Smith et al., 1991; Forsythe & Barnes-Davies, 1993; Hamann et al., 2003; Awatramani et al., 2004).

The obvious quality that separates this synapse from others in the CNS is the giant presynaptic terminal which is morphologically specialized to place all of the active zones (AZs) in a single morphologically and electrically compact structure. The apparent purpose of this specialization is to relay accurately timed inhibitory input to the MSO and LSO with minimal latency and with accurate and consistent timing (Wu & Kelly, 1993; Forsythe, 1994; Borst et al., 1995; Brew & Forsythe, 1995; Smith et al., 2000).

This branch of the sound localization circuitry seems particularly well tuned for rapid signal transmission. Interestingly, the GBCs and MNTB neurons share a number of similar characteristics such as: large thick axons, highly non-linear current-voltage (I-V) relationships, short membrane time constants, and presynaptic input via specialized calyceal nerve terminals (Morest, 1968; Oertel, 1999). These commonalities suggest that these synapses are “built” for a similar purpose within the circuit (Joris & Yin, 1998).

The calyx of Held-MNTB synapse has been particularly well studied and appears an ideal model for rapid and effective synaptic transmission (see also Hermann et al., 2007) with its numerous functional and morphological adaptations (see section 1.1.3). These adaptations are crucial as the calyx of Held-MNTB synapse places an additional relay within the inhibitory side of the sound localization circuit to both the MSO and LSO. In contrast, the excitatory pathways contain only a single synapse between the aVCN and the MSO/LSO (Fig. 1.1). Despite the additional synapse, transmission of inhibition through the system can arrive at the LSO/MSO coincidentally, or even preceding excitation (Boudreau & Tsuchitani, 1968; Tsuchitani, 1988). This rapid propagation is due to the increased rates at which synaptic transmission occurs in this pathway. For example, the calyx of Held-MNTB synapses transmits
APs twice as rapidly as most conventional mammalian central nervous system (CNS) synapses (Borst & Sakmann, 1996; Taschenberger & von Gersdorff, 2000).

Given the above, the calyx of Held-MNTB synapse appears to serve two primary functions: First, it provides a transduction point at which excitation can be converted to inhibition and a powerful signal for comparison at the LSO/MSO. Second, its specializations allow for temporal compensation of additional delays imposed by the extra relay in this pathway and the transmission of inhibition with high fidelity. These properties are crucial for the rapid and accurate localization of sound. Ultimately, the detection of predators for animals near the base of the food chain, and effectiveness in hunting of those above them, depends on this ability.

1.1.3 Interaural Level Difference & Interaural Timing Difference as a Means for the Localization of Sound.

Most mammals use two methods of sound localization depending on the frequency emitted from the source. In the auditory brainstem, two separate networks of synapses appear to have evolved to separate these processes, one that uses ILDs and one that uses ITDs to discriminate azimuthal sound source placement.

The primary integration point for the ITD system lies in the MSO where both ipsilateral and contralateral excitation from SBCs is compared along with inhibition from MNTB neurons. ITD is computed when the timing or phase of an incoming sound differs between the two ears due to a difference in distance from the source (Lord Rayleigh, 1907; Grothe, 2003). While this system is extremely accurate, able to discriminate ITDs of ~10μs in some mammals, it is fundamentally limited by the relationship between the wavelength of the source sound and the separation distance between the ears. The limit arises due to the effect that the animals head has on the scattering of the sound wave and the fact that timing differences must be detected within half the period of sounds wavelength. If the wavelength of the sound is too short, the difference in arrival time of sound at the ears contains more than one half-period and thus aliases the signal, producing a misleading image of the true sound (Joris et al., 1998; Grothe, 2003) (Fig. 1.2). It is therefore not surprising that the neurons comprising the ITD pathways show little response to higher frequency sounds (Joris et al., 1998; Tollin, 2003). MSO neurons have been found arranged in a tonotopic array which may convey Jeffress-like delay-line characteristics to this nucleus and allow for a topographic representation of azimuthal space (Jeffress, 1968; Grothe & Park, 1998; Joris et al., 1998; Grothe, 2003). The above implies that, despite the role of encoding low-frequency sound placement, discrimination of ITDs in this pathway must occur for very small differences in input timing, especially for animals with small heads. In the bat for example, ITDs of tens of microseconds have to be discriminated (Simmons et al., 1990).

Here we will focus primarily on the network of neurons involved in the localization of high-frequency sounds. High-frequency sounds are localized using ILDs which arise due to the shielding effect of the head when a sound occurs away from the midline (Rayleigh, 1877; Tollin, 2003). Low-frequency sounds travel through dense media more effectively than do high-frequency sounds, therefore the shielding effect of the head becomes more pronounced in attenuating intensity across the ears with
Figure 1.2: Schematic Illustration of ITD & ILD Sound Placement Discrimination Mechanisms. 

(A) Schematic showing detection of long wavelength, low-frequency sounds across an interaural separation distance \(d\). Accurate detection of phase difference using ITD requires that \(d\) be no longer than 0.5•wavelength of the source sound. 

(B) Illustration of “aliasing”. Source sounds with wavelengths shorter than 2•\(d\) will be perceived in an incorrect location (red source) due to an unknown number of wavelengths occurring within \(d\).

(C) Schematic of location of sound sources in space relative to excitatory ear. 

(D) Low (left panel) or high (right panel) frequency sounds are compared across the excitatory (+) and inhibitory (-) ears into a output representation from the LSO. Low-frequency sounds are compared based on the phase difference between the ears (left panel). High-frequency sounds are compared based on the SPL difference between the ears (right panel).
decreasing wavelength (Tollin, 2003). Interestingly, in both humans and cats, mid-frequency sounds (~1500-3000Hz) are not effectively localized in space presumably due to the ineffectiveness of either the ITD or ILD system at processing sound cues at the outside of their dynamic ranges (Tollin, 2003). This also lends support to the theory that two separate means are used by the auditory system to localize sound.

Sounds reaching the two ears are coded into a combination ipsilateral excitatory (SBCs) and contralateral inhibitory (MNTB) signals to the LSO that are integrated into a representation of the sound pressure level (SPL) difference between the ears. For high-frequency sounds, firing of auditory nerve fibres (ANF) onto SBCs is insufficiently rapid to code the fine structure of the input, that is, the true frequency of the sound (Johnson, 1980). However, the amplitude modulation of the signal is effectively coded at the GBCs which is the characteristic of the auditory signal that is ultimately being compared in the circuit (Joris & Yin, 1992). Sounds of different frequencies are separated by frequency-selective ANFs that innervate SBCs maintaining this tonotopic arrangement. The frequency response of the ANFs and SBCs match the range of sounds to which the ILD system must respond (>2kHz) (May & Huang, 1997). This tonotopic arrangement is maintained through the MNTB and into the LSO (Tollin, 2003).

Electrophysiological recordings performed from LSO neurons showed that when SPL is equalized between both ears, spike discharge rates are at a minimum suggesting that an excitation/inhibition comparison is necessary for the encoding of sound placement (Boudreau & Tsuchitani, 1968; Park et al., 1997). The implication of this finding is that excitation and inhibition must be arriving coincidentally under these stimulus conditions and that the increased path length and additional synapse in the inhibitory pathway must be compensated for upstream of the LSO. A simplified schematic of this concept is illustrated in Figure 1.2. The GBC-MNTB-LSO pathway decreases conduction delays in part by using large diameter axons that terminate on or near their target postsynaptic cell body (i.e. calyx of Held, also see section 1.1.2) (Cant, 1984; Smith et al., 1998). Through these specializations, the contralateral inhibitory signal is able to arrive even prior to the ipsilateral excitatory signal (Boudreau & Tsuchitani, 1968). Although the glycinergic inhibitory postsynaptic potentials (IPSPs) measured from the LSO tend to be of long duration (3-8ms, Sanes, 1990), they are effective at inhibiting excitatory postsynaptic potentials (EPSPs) only for a very brief coincidence window (1-2ms, Sanes, 1990; Wu & Kelly, 1992), meaning that very fine timing of input coincidence is resolvable by the neurons of the LSO.

Thus, in the ILD circuit, SPL differences presented at the two ears appear to be represented at the LSO, where stronger stimuli in the ipsilateral ear results in potentiated spike discharge from LSO neurons while stronger contralateral stimuli results in attenuation of the same. In combination, this allows the spike discharge pattern of the LSO to code for sound placement in the azimuth, although the subtleties of the spike discharge patterns or their higher processing is not well understood (Tollin, 2003). The biophysical and morphological design of both the GBCs and the calyx of Held-MNTB synapse are specialized to be able to transmit the inhibitory portion of this code to the contralateral LSO with maximal temporal fidelity and with high sensitivity to changes in the input signal (Joris & Yin, 1998).
1.2 THE CALYX OF HELD-MNTB SYNAPSE

1.2.1 Anatomy & Morphology.

The calyx of Held giant nerve terminal was first described in the cat by its namesake, Hans Held (Held, 1893). It is characterized by its unique morphology that envelops a significant portion of its postsynaptic MNTB target neuron (~60%) (Fig. 1.3A). Each of these giant nerve terminals arise from a single GBC via a thick myelinated axon, although some accounts of multiple calyces originating from a single axon have been reported (Morest, 1968; Spirou et al., 1990; Bergsman et al., 2004; Hoffpauir et al., 2006; Rodríguez-Contreras et al., 2006). The calyx of Held is an excitatory nerve terminal releasing glutamate onto the principal cells of the MNTB synchronously through hundreds of AZs (Sätzler et al., 2002; Taschenberger et al., 2002; Leão et al., 2005). Synchrony arises due to the uniform passive spread of APs from the heminodal region, which contains a high density of Na^+ channels, throughout the terminal which is largely void of such channels (Leão et al., 2005). Large and rapid excitatory postsynaptic currents (I_{EPSC}) are generated in response to the release of hundreds of quanta upon presynaptic AP innervation (Banks & Smith, 1992; Borst & Sakmann, 1996). The large I_{EPSC} drives fast, suprathreshold action potentials (AP) in the MNTB neuron that occur with extremely high fidelity and short refractory times (Trussell, 1997). This is due in part (discussed further below) to the rapid kinetics of presynaptic K^+ and Na^+ ion channels, the positioning of these channels, the membrane properties of the calyceal nerve terminal, and the rapid kinetics of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) amongst others (Barnes-Davies & Forsythe, 1995; Isaacson & Walmsley, 1995; Joshi et al., 2004). While these characteristics allow for the study of high frequency, high-fidelity neurotransmission at this synapse, it is its accessibility to electrophysiological experimentation that is its strongest appeal. The large surface area of the calyx of Held allows for whole-cell patch-clamp experiments to be performed with adequate space-clamp to measure the ionic currents typical of the synapse (Leão et al., 2005; Yang & Wang, 2006). In this configuration, electrical and compositional control can be gained of the presynaptic and postsynaptic neuron simultaneously, an ability unavailable at almost any other CNS synapse (Forsythe 1994; Borst et al., 1995, see also Geiger & Jonas, 2000; Southan & Robertson, 1998) (Fig. 1.3B).

1.2.2 General Development.

During the second week of postnatal development (P7-P14), and particularly around the onset of hearing (~P11), a series of robust developmental changes occur at both the pre- and postsynaptic elements of the calyx of Held-MNTB synapse that convey its ability to faithfully reproduce high frequency sounds (Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; Joshi et al., 2004; Fedchyshyn & Wang, 2005). This ability is crucial due to the importance of temporal precision in the discrimination of ILDs within the SOC (see section 1.1). The coincidence of these changes with the onset of hearing suggests that the commencement of cochlear activity is likely to initiate a number of these adaptations.
Figure 1.3: Postnatal Development of the Calyx of Held.

(A) Conceptual illustration of calyceal development at P2 (top-left panel), P5 (top-right panel), P10 (bottom-left panel), and P15 (bottom-right panel). Filipodia extend during synapse formation to contact MNTB principal cells (P2), following which, a continuous calyceal terminal begins to form (P10). Following the onset of hearing (~P12), the calyx of Held becomes fenestrated (P15). Scale bar is approximate.

(B) DIC light microscopy images taken during the patch-clamping process from a P10 synapse (left panel) and a P17 synapse (right panel). Coloured overlays show the patching electrode (green), the presynaptic calyx of Held (orange), and the postsynaptic MNTB principal cell (blue). Notice the discontinuous morphology of the calyx of Held in the P17 image.
(Futai et al., 2001; Youssoufian et al., 2005; Erazo-Fischer et al., 2007), prior to which spontaneous activity may serve to establish synaptic connections within the circuit (Beutner & Moser, 2001).

The culmination of developmental adaptations results in a number of synapse-wide changes. First, the dynamic range of the synapse is increased so that faithful APs can be transmitted at frequencies up to 1kHz at body temperature (~38°C) (Taschenberger & von Gersdorff, 2000). Second, EPSCs become larger and more rapid (Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002). Finally, synaptic depression is reduced indicating that the overall release probability ($P_r$) of the synapse decreases (Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001; Joshi & Wang, 2002). These changes to synaptic character occur due to a combination of pre- and postsynaptic adaptations which are crucial for the accurate localization of sound. Both aspects of synaptic development are discussed below.

### 1.2.3 Presynaptic Development – Morphology.

The most striking developmental change at the calyx of Held occurs in the morphology of the nerve terminal. Prior to the onset of hearing, and following initial axonal contact (~P2-3), the calyx of held envelops the MNTB neuron in a relatively continuous “spoon-like” structure (Fig. 1.3A). Rapidly following the onset of hearing (~P14-15), the calyx begins to change into a less continuous, fenestrated structure that covers a similar portion of the MNTB neuron (Morest, 1968; Kandler & Friauf, 1993; Rowland et al., 2000; Sätzler et al., 2002; Rollenhagen & Lübke, 2006). In addition, widespread myelination of the axons of both the GBCs and MNTB begins just prior to the onset of hearing (~P8) (Leão et al., 2005).

The synaptic advantage conferred by the mature morphology appears to lie in the increased kinetics by which substances can diffuse in and out of the synaptic cleft. This includes the extrusion of glutamate, which reduces the occurrence of AMPAR desensitization/saturation (see below), and the replacement of Ca$^{2+}$ ions that may be depleted during sustained periods of high-frequency activity (Borst & Sakmann, 1999b). In addition, the fenestrated structure may retain an electrically compact path through which the passive spread of APs, from the heminode to the extremities of the nerve terminal, can occur with less distortion (Leão et al., 2005). However, this morphological change also makes electrophysiological recordings from the terminal more difficult with maturation. Once the calyx has fenestrated, only the heminode is reasonably accessible to recording electrodes but yielding reliable whole-cell presynaptic recordings with good space-clamp (Fedchyshyn & Wang, 2005; Yang & Wang, 2006).

### 1.2.4 Presynaptic Development – Ion Channels.

During development, the composition of ion channels at the calyx of Held changes to allow for more rapid AP kinetics (Taschenberger & von Gersdorff, 2000; Fedchyshyn & Wang, 2005; Yang & Wang, 2006) and more effective transduction of APs into release of synaptic vesicles (SV) (Fedchyshyn & Wang, 2005; Yang & Wang, 2006). The type and densities of Na$^+$ and K$^+$ ion channels determine the depolarization and repolarization kinetics of APs respectively.
At the calyx of Held, Na\(^+\) channels are located primarily in an unmyelinated region preceding the calyceal terminal termed the heminode (Leão \textit{et al}., 2005). With maturation, the density of Na\(^+\) channels in the heminode significantly increases generating larger Na\(^+\) currents and resulting in more rapid AP depolarization kinetics. In addition, the inactivation kinetics of Na\(^+\) channels were found to increase 2-fold during development resulting in more rapid termination of the AP depolarization. Leão \textit{et al}., (2005) hypothesize that the increase in inactivation rate is due to the increased expression of the Na\(_{1.6}\) subunit, relative to another slower gating subunits. The Na\(_{1.6}\) subunit is known to have rapid activation and inactivation characteristics, which fits this hypothesis (Zhou & Goldin, 2004). Combined with the heminodal positioning of these channels, and the fenestrated morphology, these results explain the robust decrease in AP half-width (~0.5-0.3ms) observed in current-clamp recordings from the mature calyx of Held (Taschenberger & von Gersdorff, 2000, Fedchyshyn & Wang, 2005; Yang & Wang, 2006).

In conjunction with developmental changes in Na\(^+\) channels, K\(^+\) channel expression also changes (Nakamura & Takahashi, 2007). As with Na\(^+\) channels, K\(^+\) channel density increased 2-3-fold from P7 to P14 while the kinetics of the currents also increase by a similar degree. Both K\(_{1,1}\) and K\(_{3,3}\) channel subtype densities increased with development, however they were localized to different portions of the terminal, with K\(_{3,3}\) channels being disperse throughout the terminal and K\(_{1,1}\) channels being expressed primarily at the heminode (Dodson \textit{et al}., 2003). The placement of K\(_{3,3}\) channels within the terminal, and their more rapid activation kinetics, implies that they are primarily responsible for the repolarization of the AP (Nakamura & Takahashi, 2007). The function of K\(_{1,1}\) channels appears to suppress aberrant AP firing which may explain their positioning at the heminode (Ishikawa \textit{et al}., 2003). The increase in kinetics of both channel types most likely arises from increased expression of faster channel subtypes within each group (i.e. K\(_{3,3}\)/4 for K\(_{3,3,1/2}\)). Combined, the increase in K\(^+\) current density and activation kinetics suggests that this developmental adaptation works in concert with changes in Na\(^+\) channel properties to narrow the AP waveform and increase the presynaptic response range of the calyx of Held (Leão \textit{et al}., 2005; Nakamura & Takahashi, 2007).

The primary function of presynaptic APs is to gate voltage-gated calcium channels (VGCCs) thereby initiating and shaping the [Ca\(^{2+}\)]\textsubscript{i} signal. This Ca\(^{2+}\) signal determines if, and with what kinetics, SVs are released (Augustine, 1990; Borst & Sakmann, 1999; Bollmann & Sakmann, 2005; Taschenberger \textit{et al}., 2005; Fedchyshyn & Wang, 2005; Yang & Wang, 2006; see Chapter 5). During development, the composition of VGCCs that are present, and that mediate release of SVs, changes at the calyx of Held. Prior to P11, N and P/Q-type VGCCs are equally efficacious in triggering SV release and cooperate in that capacity (Iwasaki & Takahashi, 1998; Wu \textit{et al}., 1999; Fedchyshyn & Wang, 2005). A measurable R-type component is also present but mediates a relatively small portion of the overall calcium current (I\textsubscript{Ca}) and an even smaller portion of release (I\textsubscript{EPSC}) (Wu \textit{et al}., 1998; Iwasaki \textit{et al}., 2000). With development, and as early as P11, N-type VGCCs begin to leave the AZ, mediating less and less SV release, approaching zero efficacy by approximately P13 (Iwasaki & Takahashi, 1998; Fedchyshyn & Wang, 2005). During this time, R-type mediated I\textsubscript{Ca} and I\textsubscript{EPSC} also decreases to immeasurable levels (Iwasaki & Takahashi, 1998). In contrast, P/Q-type VGCCs increase their contribution mediating almost all SV release from the terminal by P13 (Iwasaki & Takahashi, 1998; Fedchyshyn & Wang, 2005). The
above trends are typical amongst many neurons throughout the CNS (Iwasaki et al., 2000). P/Q-type VGCCs, although similar in kinetics and properties to N-type VGCCs, exhibit \( I_{\text{Ca}} \) facilitation during repetitive stimulation (Tsujimoto et al., 2002; Inchauspe et al., 2004; Ishikawa et al., 2005; Inchauspe et al., 2007). \( I_{\text{Ca}} \) facilitation is thought to arise through the action of neuronal Ca\(^{2+}\) sensor 1 (NCS-1) specifically on P/Q-type VGCCs (Tsujimoto et al., 2002). Since P/Q-type VGCCs are the subtype exclusively mediating SV release into maturity, it is crucial to understand by what means these VGCCs are differentially modulated and thus why they are advantageous for high-fidelity transmission with maturity. The recent availability of P/Q-type null mice has enabled preliminary work that has begun to address these issues (Inchauspe et al., 2004; Ishikawa et al., 2005; Inchauspe et al., 2007).

1.2.5 Presynaptic Development – Spatial Localization of VGCCs and SVs.

As described above, one of the developmental characteristics of the calyx of Held is a narrowing of the half-width of presynaptic APs. Narrower APs reduce the time of maximum activation voltage for VGCCs, in turn lowering their cumulative \( I_{\text{Ca}} \) across the terminal (Pattillo et al., 1999). One paradox at the calyx of Held surrounded how the magnitude of SV release could be maintained, or even increased, while the \( I_{\text{Ca}} \) generating it decreased. Recent studies by Fedchyshyn & Wang (2005) and Yang & Wang (2006) have concluded that SV release is maintained due to a tighter coupling between VGCCs and SVs. Thus, a given \( I_{\text{Ca}} \) triggers release more efficaciously in mature synapses than in immature ones.

Around the onset of hearing, P/Q-type VGCCs begin to tighten their coupling with SVs while, at the same time, N-type VGCCs recede from the AZ (Fedchyshyn & Wang, 2005). This subsynaptic adaptation reduces the requirement for many VGCCs to contribute in generating a suprathreshold \( I_{\text{Ca}} \). Thus, a decrease in AP half-width decreases \( P_r \), while tightening the coupling between VGCCs and SVs increases \( P_r \). The net result is an approximate maintenance of synaptic output with maturation (Fedchyshyn & Wang, 2005; Yang & Wang, 2006). The further effects of changes in VGCC-SV coupling with development are described in Chapter 6. In addition, altering the number of VGCCs contributing to the Ca\(^{2+}\) signal and their distance from the SVs has implications for the efficacy of endogenous buffers and for the kinetics of the Ca\(^{2+}\) sensor itself. These issues are discussed below.

1.2.6 Presynaptic Development – Calcium Buffers.

One of the key factors determining the shape and spread of Ca\(^{2+}\) signals generated by the opening of VGCCs are the kinetics and concentrations of Ca\(^{2+}\) buffers (Naraghi & Neher, 1997; Meinrenken et al., 2002). To date, only two Ca\(^{2+}\) binding proteins (CBP) have been developmentally characterized at the calyx, parvalbumin and calretinin (Felmy & Schneggenburger, 2004). Both CBPs were found to increase in expression with development however, expression of calretinin was inconsistent with a primary role in Ca\(^{2+}\) buffering as it was found only in a certain subset of calyces within the MNTB (Felmy & Schneggenburger, 2004). Subsequent studies have found that parvalbumin is the endogenous buffer most likely to shape the Ca\(^{2+}\) signal at the immature calyx of Held (Müller et al., 2007). Given the developmental increase in parvalbumin expression uncovered by Felmy & Schneggenburger (2004), it is likely that the resultant increased buffer capacity in mature synapses
serves to further restrict the spread of Ca$^{2+}$ domains (Naraghi & Neher, 1997). Given a tighter coupling between VGCCs and SVs, this may serve to spatially isolate Ca$^{2+}$ signals at one release site from its neighbors, leading to independence of release sites (see similar arguments for synaptic independence, Overstreet & Westbrook, 2003; Barbour, 2001).

1.2.7 Presynaptic Development – Quantal Properties.

A significant body of work has surrounded the developmental changes that occur to the fundamental units of quantal release at the calyx of Held. As outlined above, with maturation, the efficiency of transmission (input/output) increases. While a portion of that increase is due to the more effective coupling of VGCCs to SVs (Fedchyshyn & Wang, 2005), others have hypothesized that the increased efficiency may arise due to fundamental changes to the quantal building blocks of the $I_{\text{EPSC}}$. $I_{\text{EPSC}}$ is a function of the average quantal current amplitude ($i$), the total number of independent release sites ($N$), and the probability of release ($P_R$) forming the classic equation $I_{\text{EPSC}} = P_R \cdot N \cdot i$ (see Sakaba et al., 2002). Each variable within this equation is a potential source of developmental modification.

Quantal size ($i$) has been reported as both consistent and variable with development. In immature synapses (~P8), average quantal size ranges between 30 and 40pA (Schneggenburger et al., 1999; Meyer et al., 2001). With development, quantal size appears to increase to ~50pA when a sufficient separation in experimental age groups is allowed (>P14) (Yamashita et al., 2003; Taschenberger et al., 2005; Erazo-Fischer et al., 2007). Earlier studies reported no such change in quantal size but also used larger ranges and more closely spaced age groups for analysis (Chuma & Ohmori, 1998, Taschenberger & von Gersdorff, 2000). If it is assumed that the quantal size increases slightly at some point during development, then this could also partially explain the increased synaptic efficacy observed in mature synapses. Increases in quantal size could come as a result of increased SV diameter or increased packaging of glutamate into the SVs, the later appearing the most likely cause (Wu et al., 2007). The caveat to this is that the apparent increased quantal size could arise due to increased expression of postsynaptic AMPARs, provided that some degree of saturation occurs prior to adding more AMPARs. AMPARs exhibit increased developmental clustering opposing presynaptic release sites, which may be crucial for sustaining accurate reporting of presynaptic release during high-frequency activity (Hermida et al., 2006). However, single quanta do not saturate AMPARs in the immature calyx of Held-MNTB synapse suggesting that the quantal size measured via miniature $I_{\text{EPSC}}$ (mEPSC) is likely accurate throughout development (Yamashita et al., 2003).

As the calyx of Held matures, the number of morphologically defined AZs approximately doubles while the area of each AZ decreases significantly (Taschenberger et al., 2002). While these changes may seem to oppose each other in terms of defining the number of release sites ($N$), the authors also measured a significant increase in the number of docked SVs suggesting that the number of release sites is in fact increasing with development overall. An increase in the number of release sites also increases the size of the readily releasable pool (RRP) of SVs. In line with this assumption, a 2-fold developmental increase in the size of the RRP has been observed at the calyx of Held (Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001; Taschenberger et al., 2002; Youssoufian et al., 2005).
The size of the RRP becomes of particular concern when evaluating a synapse's ability to sustain SV output during high-frequency activity. This will be discussed in greater detail below. The increase in AZ number is also consistent with the observed increase in miniature excitatory postsynaptic currents (mEPSC) frequency with development (Iwasaki & Takahashi, 2001; Joshi et al., 2004).

The final component of the quantal hypothesis is \( P_r \). This synaptic property influences almost all aspects of synaptic function from determining the magnitude of quantal output during single stimuli, to pacing the usage of SVs from within the RRP, and shaping the patterns of short-term plasticity (STP). The measured \( P_r \) at the calyx of Held-MNTB synapse decreases approximately 2-fold around the onset of hearing (Iwasaki & Takahashi, 2001; Taschenberger et al., 2002; Taschenberger et al., 2005). The decrease in \( P_r \) is due primarily to the reduction in the width of APs, which significantly reduces the \( I_{Ca} \).

Since \( I_{EPSC} \) is a highly non-linear function of \( I_{Ca} \) \( (I_{EPSC} \propto (I_{Ca})^\alpha) \), these decreases are amplified causing significant reductions in \( P_r \) (Dodge & Rahamimoff, 1967; Taschenberger & von Gersdorff, 2000). However, reductions in AP width are compensated for by increasing coupling efficacy of \( Ca^{2+} \) influx with SV release, thereby tempering the decrease in \( P_r \) overall (Fedchyshyn & Wang, 2005).

In combination, these maturations to the fundamental quantal properties of transmission aid the calyx of Held-MNTB synapse in acquiring the high-frequency and high-fidelity characteristics that it is renowned for.

### 1.2.8 Presynaptic Development – Other Receptors & Ion Channels.

A number of less prominent receptors and ion channels are also present at the calyx of Held-MNTB synapse, including Cl\(^{-}\) channels, \( Ca^{2+} \)-activated K\(^{+}\) channels, cannabinoid receptors (CBR), etc... (Kushmerick et al., 2004; Price & Trussell, 2006) To date, no developmental characterization of these synaptic elements has been performed. In addition, certain synaptic receptors and channels are expressed early in development but are gradually removed from the synapse with development. The actions of these receptors are reasonably well understood in the immature synapse, however, their absence from the mature synapse implies that their necessity is transient.

Expression of metabotropic glutamate receptors (mGluRs) at the presynaptic terminal consists of all three classes (I, II, & III), although only groups II and III appear to have significant inhibitory roles at the immature calyx of Held (Kushmerick et al., 2004; Renden et al., 2005). mGluRs detect elevations in glutamate concentrations, either in the synaptic cleft (mGluR4) or extrasynaptically (mGluR2/3 & 8) and convert this into inhibition via G-protein inhibition of P/Q-type VGCCs (Takahashi et al., 1996). Although somewhat different in mouse and rat, expression of groups II and III mGluRs is absent from the mouse calyx of Held by ~P16, while the developmental fate of group I mGluRs is unknown (Kushmerick et al., 2004; Renden et al., 2005). It is hypothesized that the presence of mGluRs at the immature calyx of Held allows for negative feedback control of glutamate output during periods of significant SV release, thereby reducing the rate of expenditure of the RRP (Renden et al., 2005). In addition, activation of mGluRs could reduce glutamate output in an effort to reduce potential AMPAR desensitization and saturation, however, the extent to which mGluRs are actually involved in attenuating synaptic output is unclear (von Gersdorff et al., 1997). Interestingly, the proportional expression of P/Q-type VGCCs...
increases with development, which would provide a very effective target for inhibition if mGluRs were still present.

Another G protein-coupled receptor (GPCR) that has similar developmental characteristics as mGluRs is the adenosine 1 receptor (A1R). However, unlike mGluRs, A1Rs target inhibition to both N- and P/Q-type VGCCs at this synapse (Wu & Saggau, 1994; Kimura et al., 2003). Attenuation of $I_{Ca}$ works ultimately through the same mechanism as mGluRs and GABA$_B$ receptors (discussed below) to reduce synaptic output. Adenosine is thought to be released in the form of adenosine triphosphate (ATP) along with glutamate, which in turn activates presynaptic A1Rs when the adenosine concentration is elevated (>~1μM) (Kimura et al., 2003). However, as with mGluRs, A1Rs appear to decrease in expression during the second postnatal week, implying that their role in inhibiting release during high-frequency stimulation is necessary only early in synaptic development (Kimura et al., 2003).

$I_{Ca}$ from immature calyces appears to also be inhibited by activation of noradrenaline (NA) receptors, although whether a specific subtype of VGCC is targeted is unknown (Leão & von Gersdorff, 2002). As above, activation of α2-adrenergic receptors with NA inhibits $I_{Ca}$, which in turn decreases synaptic output, an effect that is absent from calyces above P14 (Leão & von Gersdorff, 2002). Interestingly, reduction of synaptic output by NA application, increased excitability of MNTB neurons, due to a decreased activation of N-methyl-D-aspartic acid receptors (NMDAR), and reduced the associated postsynaptic plateau current (Leão & von Gersdorff, 2002).

5-Hydroxytryptamine (5-HT) or serotonin receptors also show a developmental decrease in their effectiveness in attenuating transmitter release during the second postnatal week (Mizutani et al., 2006). Activation of specifically the 5-HT$_{1B}$ receptor subtype activates a G protein mediated signaling pathway similar to those above (Mizutani et al., 2006).

Finally, activation of γ-aminobutyric acid B receptors (GABA$_B$) acts on VGCCs attenuating $I_{Ca}$ and reducing synaptic output. The mechanism of this inhibition has been well studied at the calyx of Held and has been found to rely on G$_{oβγ}$ subunits acting to specifically slow the activation kinetics of $I_{Ca}$ (Barnes-Davies & Forsythe, 1994; Takahashi et al., 1998; Kajikawa et al., 2001). Expression levels of GABA$_B$Rs varies during development between experimental systems. In rats, GABA$_B$R expression levels decrease only slightly during the second postnatal week (Renden et al., 2005). In mice, GABA$_B$Rs show a more significant reduction (~50%) in their expression levels by the third postnatal week (Renden et al., 2005). In addition, activation of GABA$_B$Rs appears to slow recruitment of SVs into the RRP by reducing cyclic adenosine monophosphate (cAMP) (Sakaba & Neher, 2003). Currently, it is unknown whether GABA$_B$Rs ever completely disappear from the calyx of Held; however, their decreasing effectiveness in inhibiting $I_{Ca}$ with maturation suggests that the role of these receptors is more influential in immature synapses.

The many similarities in the above mechanisms of synaptic modulation, and their developmental expression patterns, suggests that the immature synapse is a higher priority target for a variety of modulatory signaling cascades. Many of these modulatory pathways appear to be “pruned out” directly following the onset of hearing in the second postnatal week implying that the onset of sensory activity may drive one or any number of these changes. Finally, each of these receptor pathways acts on the
most non-linear element influencing SV release ($I_{Ca}$). In this way, relatively small effects are amplified to have significant impact on the firing of the postsynaptic neuron early in development of this synapse.

1.2.9 Postsynaptic Development – AMPARs.

Concurrent with many of the presynaptic adaptations described above, a number of postsynaptic characteristics also change with development in order to effectively receive the refined synaptic output of the mature calyx of Held. With development, the expression of different ionotropic glutamate receptors is one means through which this occurs. Reception of glutamatergic output occurs through AMPA, NMDA, and kainate receptors spanning the postsynaptic membrane. At the calyx of Held-MNTB synapse, AMPARs mediate the fast response to glutamate while NMDARs mediate a slower response discussed below (Barnes-Davies & Forsythe, 1995).

Multimeric AMPARs consist of GluR1-4 subunits, the choice of which determines many of the functional properties of the channel. GluR1/2 subunits impart slower gating kinetics on the channel than do the GluR3/4 subunits. In addition, GluR1-4 have both “flip” and “flop” isoforms of which flop are faster gating isoforms (Mosbacher et al., 1994; Geiger et al., 1995; Koike et al., 2000). The GluR2 subunit is somewhat unique in that its presence restricts the conductance of $Ca^{2+}$ ions through the channel (Hollmann et al., 1991; Sommer et al., 1991; Burnashev et al., 1992; Jonas et al., 1994).

With development, the relative expression of GluR3/4 containing receptors increases relative to GluR1 (Joshi et al., 2004; Koike-Tani et al., 2005), while GluR2 containing AMPARs appear to be absent at this synapse throughout development (Joshi et al., 2004). These changes in AMPAR subtypes serve to increase both the rise and decay of $I_{EPSC}$ due to their more rapid gating kinetics. This postsynaptic modification allows for more rapid generation of APs at the MNTB (Joshi et al., 2004; Koike-Tani et al., 2005). In addition, the kinetics of $I_{EPSC}$ has implications for the rate and extent to which desensitization and deactivation occurs (Koike-Tani et al., 2005). Deactivation and desensitization are believed to shape the decay kinetics of $I_{EPSC}$ (Takahashi, 2005). Depending on which channel subtypes are dominant, and depending on the rate of glutamate clearance from the synaptic cleft, both of these mechanisms have been shown to underlie this property of AMPAR-$I_{EPSC}$. When glutamate clearance is fast, deactivation shapes the decay of $I_{EPSC}$, while desensitization performs the same function when clearance is slow (Trussell et al., 1993; Silver et al., 1996; Hansen et al., 2007).

At the calyx of Held-MNTB synapses, these mechanisms are particularly relevant as the clearance rates of substances from the synaptic cleft is significantly increased as fenestration of the terminal occurs (see above). While this morphological change would favour deactivation as a means of defining the decay of AMPAR-$I_{EPSC}$, the prevalence of fast desensitizing GluR4 subunits would suggest that desensitization would become more efficacious at determining the decay of $I_{EPSC}$ with development. In fact, both deactivation and desensitization contribute to decay of AMPA-$I_{EPSC}$ at the MNTB, with the proportional contribution favouring primarily deactivation with development (Koike-Tani et al., 2005). In combination with a developmental switch to the faster gating GluR3/4 flop subunits, the observed increase in AMPAR-$I_{EPSC}$ kinetics can be fully explained (Taschenberger & von Gersdorff, 2000; Joshi et al., 2004; Koike-Tani et al., 2005; Takahashi, 2005).
A decrease in the nature of AMPAR desensitization and saturation is particularly important when considering the effect of desensitization accumulation during sustained high-frequency activity. Desensitized AMPARs effectively produce a partially “deaf” synapse that is unable to match the dynamic range of presynaptic output. This is particularly obvious at the immature calyx of Held where quantal size may be underestimated by as much as 2.5 times due to desensitization (Scheuss et al., 2002). The implications of this underestimation are that the rate of short-term depression (STD) appears more rapid than it actually is, and may even mask the occurrence of short-term facilitation (STF) (Wong et al., 2003). AMPAR desensitization can then affect estimates of the number of release sites (N), number of AZs, STP kinetics, RRP size, GluR subtype determination, and P, depending on the experimental and analytical approach (see Neher & Sakaba, 2001). As a result, AMPAR saturation/desensitization must be carefully considered especially when glutamate clearance is slowed as in immature synapses.

The increase in AMPAR-\(I_{\text{EPSC}}\) kinetics with development is crucial for sustaining rapid and reliable generation of APs from the MNTB neuron. By ensuring a rapid return to a deactivated state, mature synapses allow for the rapid recycling of their AMPARs back to conduction-ready states, ultimately allowing for faithful passing of more rapidly cycling presynaptic input in the mature synapse (Joshi et al., 2004; Koike-Tani et al., 2005; Takahashi, 2005). These postsynaptic adaptations allow for mature MNTB neurons to be a more faithful reproducer of presynaptic input and, when combined with the presynaptic changes outlined above, result in a higher fidelity neuron with expanded dynamic range that matches that of the calyx of Held.

1.2.10 Postsynaptic Development – NMDARs.

The importance of NMDARs in synaptic plasticity has been well characterized in countless review articles. However, at the calyx of Held-MNTB synapse, forms of NMDAR-mediated long-term plasticity have been elusive, although some forms of post-tetanic potentiation have been characterized which appear to be presynaptic in origin (Habets & Borst, 2005; Korogod et al., 2005; Habets & Borst, 2006; Habets & Borst, 2007; Korogod et al., 2007). This finding makes the significant NMDAR-mediated current, observed in the immature calyx of Held, curious in its function.

NMDARs exist as heteromers of a necessary NR1 subunit and subunits of the NR2A-D type, the latter being the determinant of the biophysical properties of the channel and its localization (Takahashi et al., 1996; Mori et al., 1998). NMDARs are unique in that they act as coincidence detectors for the neurotransmitters glutamate and glycine (Johnson & Ascher, 1987), but also require significant depolarization in order to alleviate blockage of the channel pore by Mg\(^{2+}\) ions (MacDonald & Wojtowicz, 1980; Nowak et al., 1984). In this sense, under physiological conditions, NMDARs preferentially detect the occurrence of intense synaptic activity. In addition, NMDARs also flux Ca\(^{2+}\) ions, which trigger some of the processes involved in expression of various forms of LTP (Malenka et al., 1989; Nicoll & Malenka, 1999).

Prior to the onset of hearing (~P11), NMDARs exist in high densities in postsynaptic MNTB neurons and mediate large NMDA-\(I_{\text{EPSC}}\) under appropriate conditions (Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002). However, their expression levels begins to decline after this period until
NMDA-\(I_{\text{EPSC}}\) are barely detectable by P16 (Futai et al., 2001; Joshi & Wang, 2002). In addition, NMDA-\(I_{\text{EPSC}}\) show a developmental increase in their decay kinetics. This is hypothesized to arise from a switch between NR2B-C to the faster NR2A subunit or from the increased diffusion rate of glutamate out of the calyx with morphological development (Takahashi et al., 1996; Joshi & Wang, 2002). Interestingly, both of the developmental changes observed with NMDARs require sensory activity for initiation (Futai et al., 2001, Youssoufian et al., 2005). Recent evidence from the calyx of Held-MNTB synapses suggests that it is the co-activation of NMDARs and mGluRs (group1) that leads to the down-regulation of NMDARs coincident with the onset of activity (Joshi et al., 2004).

The loss of NMDA-\(I_{\text{EPSC}}\) serves an important role at the calyx of Held-MNTB synapse. As a result of the slow deactivation kinetics of NMDARs, prolonged “plateau” currents arise during repetitive activity. This prolonged potential results in non-phase-locked AP firing and progressive inactivation of Na\(^{+}\) channels, restricting the MNTB neurons’ ability to faithfully reproduce presynaptic APs (Taschenberger & von Gersdorff, 2000; Joshi et al., 2002). When the plateau potential is absent, either through pharmacological block of NMDARs or through activity-dependent reduction, the incidence of aberrant AP firing or postsynaptic AP generation failures dramatically decreases (Taschenberger et al., 2000; Joshi & Wang, 2002; Joshi et al., 2007). These findings suggest that the loss of NMDARs from the postsynaptic neurons is a crucial step in the acquisition of high-fidelity responsiveness in the postsynaptic MNTB neuron.

While it appears clear that their loss is necessary, the function of NMDARs in the immature synapse also remains elusive. In the absence of identifiable forms of plasticity that are dependent on these receptors, their potential function will remain to be elucidated by future experimentation.

1.3 HIGH-THRESHOLD VOLTAGE-GATED CALCIUM CHANNELS

1.3.1 Types, Subunits, Structure, & Localization – The \(\alpha_1\) Subunit.

Voltage-gated Ca\(^{2+}\) channels (VGCCs) of various types are crucial for coupling voltage signals to biological responses throughout the CNS (Hoffmann et al., 1994; Jones, 1998; Catterall, 2000). Of particular importance is the role VGCCs play in excitation-secretion coupling in the CNS and excitation-contraction coupling in muscle (Jones, 1998). Their wide range of properties and various forms of modulation allow VGCCs to serve diverse signalling functions at the organism, system, cellular, and subcellular levels.

To date, nine separate types of neuronal VGCCs have been identified and are classified using both an alphabetical and numerical classification system (Hoffmann et al., 1994; Jones, 1998; Ertel et al., 2000) (Table 1.1). Throughout this dissertation we have referred to VGCCs by the alphabetical classification, which include L, N, P, Q, R, and T-types. The various VGCCs can be classed in a broad sense into high-threshold (L, N, P, Q, and R) and low-threshold (T) varieties that refers to the voltage-threshold at which the channels are activated (Jones, 1998; Catterall, 2000). The nine neuronal VGCCs...
<table>
<thead>
<tr>
<th>Alpha-Numeric Nomenclature</th>
<th>Alphabetical Nomenclature</th>
<th>Greek Nomenclature</th>
<th>Principle Localization</th>
<th>Primary Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsubscript{v}1.1</td>
<td>L-Type</td>
<td>\alpha\textsubscript{1S}</td>
<td>Skeletal Muscle</td>
<td>DHP</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2</td>
<td>L-Type</td>
<td>\alpha\textsubscript{1C}</td>
<td>Cardiac &amp; Smooth Muscle; CNS; Respiratory, Endocrine Systems</td>
<td>DHP</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.3</td>
<td>L-Type</td>
<td>\alpha\textsubscript{1D}</td>
<td>CNS; Endocrine, Reproductive &amp; Auditory Systems</td>
<td>DHP</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.4</td>
<td>L-Type</td>
<td>\alpha\textsubscript{1F}</td>
<td>Visual System</td>
<td></td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.1</td>
<td>P/Q-Type</td>
<td>\alpha\textsubscript{1A}</td>
<td>CNS; Auditory &amp; Adrenal Systems</td>
<td>\omega-agatoxin-IVA</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.2</td>
<td>N-Type</td>
<td>\alpha\textsubscript{1B}</td>
<td>CNS</td>
<td>\omega-conotoxin-GVIA</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.3</td>
<td>R-Type</td>
<td>\alpha\textsubscript{1E}</td>
<td>CNS; Auditory, Visual, Cardiac &amp; Adrenal Systems</td>
<td>SNX-482</td>
</tr>
</tbody>
</table>

Table 1.1: High-Threshold Voltage-Gated Calcium Channel Subtypes, Nomenclature, & Pharmacology.
Adapted from Hoffmann et al., 1994; Birbaumer et al., 1998; Jones, 1998; Meir et al., 1999; Catterall, 2000; Fischer & Bourque, 2001. DHP - Dihydropyridine.
arise due to alternative splicing of the six types listed and are denoted by the second digit in their numerical classification (Table 1.1) (Catterall, 2000). The first digit refers to the type of \( \alpha_1 \) subunit contained within the channel and appears to largely determine the properties of the channel (Catterall, 2000; Evans & Zamponi, 2006). In addition to the \( \alpha_1 \) subunit, VGCCs may have auxiliary \( \beta, \alpha_2-\delta, \) and \( \gamma \) subunits that act in a variety of functional roles described in greater detail below (Fig. 1.4). However, the various combinations of VGCC subunits impart different voltage dependencies, activation and inactivation kinetics, localization, and expression levels establishing their importance in the diverse characteristics of VGCCs.

The various types of VGCCs are distinguished largely by their sensitivity to various pharmacological compounds or organic toxins. N-type VGCCs are readily blocked by \( \omega \)-conotoxin-GVIA, L-type VGCCs are highly sensitive to blockade by dihydropyridines (DHP), P & Q-type VGCCs are distinguishable based on their respectively high and low-affinity for \( \omega \)-agatoxin-IVA, while R-type VGCCs are characterized by their insensitivity to any of the above (McCleskey et al., 1987; Mintz et al., 1992; Randall & Tsien, 1995). These differences in sensitivity are most likely the result of sections of highly diverse amino acid sequences within the \( \alpha_1 \) subunits. N, P, and Q-type VGCCs share 70% sequence identity. However, L-type and T-type share only 40% and 25% sequence identity with these VGCCs respectively (Catterall, 2000). In relation to this dissertation, only the high-threshold VGCCs, expressed highly in the CNS, of the P, Q, N and R-types and their associated \( \alpha_1 \) subunits will be considered further.

1.3.2 Types, Subunits, Structure, & Localization – The \( \beta \) Subunit.

\( \beta \) subunits interact primarily with the alpha-interacting domain (AID) of all high-threshold \( \alpha_1 \) subunits, secondarily with sites identified on the C-terminus of P/Q-type and R-type VGCCs, and N-terminus of P/Q-type and N-type VGCCs (Tareilus et al., 1997; Birnbaumer et al., 1998; Walker et al., 1999; De Waard et al., 1994; Dolphin, 2003). There have been four \( \beta \) subunits identified and numbered 1-4 after the genes that encode them (Castellano et al., 1993a, Castellano et al., 1993b; Perez-Reyes et al., 1992; Birnbaumer et al., 1998). Their effect on \( I_{Ca} \) depends both on the type of \( \beta \) subunit and which \( \alpha_1 \) subunits they are in association with (Table 1.2) (Hoffmann et al., 1994; Birnbaumer et al., 1998; Arikkath & Campbell; 2003). One of the primary functions of the \( \beta \) subunit is to increase the surface expression and targeting of \( \alpha_1 \) subunits to the cell membrane and increase whole-cell current (Williams et al., 1992a; Williams et al., 1992b). In addition, association of \( \beta \) subunits alter the biophysical properties of the VGCC producing, in general, a hyperpolarizing-shift in the voltage-dependence of activation and inactivation which is believed to arise due to a more effective movement of the channel voltage sensor (Singer et al., 1991; Neely et al., 1993; Birnbaumer et al., 1998).

The \( \beta_4 \) subunit is most commonly associated with P/Q-type VGCCs whereas the \( \beta_3 \) subunit is found most often associated with N-type VGCCs in the CNS and is clearly an integral partner in the proper functioning of VGCCs (De Waard & Campbell, 1995; Scott et al., 1996).
Figure 1.4: Molecular Structure of Voltage-Gated Calcium Channels.
Voltage-gated calcium channels (VGCCs) are comprised of a pore-forming \( \alpha \) subunit, a modulatory \( \beta \) subunit, and two other auxiliary subunits, the \( \gamma \) and \( \alpha,\delta \) subunits. VGCCs are classified according to the type of \( \alpha 1 \) subunit they express. While the \( \alpha 1 \) subunit is the minimal unit required to produce a functional channel, the \( \beta \) subunit is generally required for complete expression and normal biophysical channel behaviour. Neither the \( \beta \) or \( \alpha \) subunits are membrane-bound being cytosolic and extrasynaptic respectively, all other subunits contain at least one membrane-spanning section. The \( \alpha \) subunit is comprised of four homologous domain (I–IV) which together form the channel pore and contain the voltage-gate for the channel (S4). Adapted from Hoffmann et al., 1994; Catterall, 2000; Anikkath & Campbell, 2003; Evans & Zamponi, 2006; Lisman et al., 2007.
1.3.3 Types, Subunits, Structure, & Localization – The $\alpha_2\delta$ Subunit.

The $\alpha_2\delta$ subunits are auxiliary subunits that bind to each other through a disulphide linkage (Takahashi et al., 1987). The transmembrane $\delta$ portion of the subunit anchors the extracellular $\alpha$ portion (Hoffmann et al., 1994; Arikkath & Campbell, 2003; Klugbauer et al., 2003). Their interaction point with the $\alpha_1$ subunit is at the extracellular loop on domain III which has varying effects on the biophysical properties of the channel (Table 1.2) (Arikkath & Campbell, 2003). There are four genes that code for the four $\alpha_2\delta$ subunits, numbered 1-4 (Ellis et al., 1988; Klugbauer et al., 1999; Qin et al., 2002). Like the $\beta$ subunits, $\alpha_2\delta$ subunits appear to aid in establishing appropriate level of surface expression of $\alpha_1$ subunits (Arikkath & Campbell, 2003; Klugbauer et al., 2003). In addition, $I_{Ca}$ density and activation/inactivation kinetics can be increased by the presence of an $\alpha_2\delta$ subunit (Mori et al., 1991; Singer et al., 1991; Felix et al., 1997; Arikkath & Campbell, 2003). Interestingly, the effects of both $\alpha_2\delta$ and $\beta$ subunits appear to be complimentary in that the presence of one enhances the modulatory impact of the other (Felix et al., 1997; Mori et al., 1991; Klugbauer et al., 1999; Qin et al., 2002).

1.3.4 Types, Subunits, Structure, & Localization – The $\gamma$ Subunit.

Eight forms of the $\gamma$ subunit have been identified ($\gamma_{1-8}$) however only two have been found associated with the VGCCs common in the CNS (i.e. P/Q and N-types) being $\gamma_2$ and $\gamma_3$ respectively (Kang et al., 2001). The other $\gamma$ subunits are generally located outside the CNS (see Arikkath & Campbell, 2003). One interesting property of the $\gamma$ subunit is that it is also found associated with AMPARs, which appears to serve a trafficking role (Chen et al., 2000; Kang et al., 2001; Tomita et al., 2003). Unlike $\beta$ subunits, and to a lesser extent $\alpha_2\delta$ subunits, $\gamma$ subunits are not crucial for the functionality of VGCCs but serve a modulatory role (see Arikkath & Campbell, 2003; Klugbauer et al., 2003). Both the $\gamma_2$ and $\gamma_3$ subunits appear to increase the inactivation kinetics of P/Q and N-type VGCCs due to a hyperpolarizing shift in the inactivation voltage-dependence (Letts et al., 1998; Klugbauer et al., 2000). The $\gamma_2$ subunit has also been shown to have an inhibitory effect on the channel current (Klugbauer et al., 2000; Kang et al., 2001) (Table 1.2).

1.3.5 Structure of VGCC Subunits.

As described above, functional VGCCs contain a mix of $\alpha_1$, $\beta$, $\alpha_2\delta$, and $\gamma$ subunits. The $\alpha_1$ subunit consists of four homologous domains which comprise the pore forming unit, similar to that of the $\alpha Na^+$ channel subunit (Tanabe et al., 1987; Hoffmann et al., 1994; Reuter, 1996; Catterall, 2000). Interestingly, each of the four pore forming units shares remarkable similarity to the shaker $K^+$ channel (Birnbaumer et al., 1998). Each domain contains six transmembrane segments (S1-6), with the S4 domain conferring the voltage-sensitivity of the channel and S5/6 being responsible for ion selectivity and antagonist binding (Hoffmann et al., 1994; Jones, 1998; Catterall, 2000). The linkage loops between domains contain a number of binding sites that are acted upon by a number of modulatory pathways (discussed below).
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Other Names</th>
<th>Principle Localization</th>
<th>Binding on $\alpha_1$</th>
<th>Effect on VGCC Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$</td>
<td>CaB1</td>
<td>Skeletal Muscle; CNS</td>
<td>I-II Linker</td>
<td>Increase $I_{\text{Ca}^\text{to}}$, Activation/inactivation, trafficking $\alpha_1.1$ to membrane</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>CaB2</td>
<td>CNS; Cardiac &amp; Respiratory Systems</td>
<td>I-II linker N/C-Terminus</td>
<td>Increase $I_{\text{Ca}^\text{to}}$, Activation/inactivation, trafficking $\alpha_1.4$ to membrane</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>CaB3</td>
<td>Smooth Muscle; CNS; Cardiac &amp; Respiratory Systems</td>
<td>I-II Linker</td>
<td>Increase $I_{\text{Ca}^\text{to}}$, Activation/inactivation, trafficking of $\alpha_1$ to membrane</td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>CaB4</td>
<td>CNS</td>
<td>I-II Linker N/C-Terminus</td>
<td>Increase $I_{\text{Ca}^\text{to}}$, Activation/inactivation, trafficking of $\alpha_1$ to membrane</td>
</tr>
<tr>
<td>$\gamma_1$</td>
<td>CaG1</td>
<td>Skeletal Muscle</td>
<td>---</td>
<td>Inhibition, Activation/Inactivation</td>
</tr>
<tr>
<td>$\gamma_2$</td>
<td>CaG2</td>
<td>CNS</td>
<td>---</td>
<td>Inhibition, Activation/Inactivation, AMPAR Trafficking</td>
</tr>
<tr>
<td>$\gamma_3$</td>
<td>CaG3</td>
<td>CNS</td>
<td>---</td>
<td>Activation/Inactivation Kinetics</td>
</tr>
<tr>
<td>$\gamma_4$</td>
<td>CaG4</td>
<td>Spinal Cord; Cardiac &amp; Respiratory Systems</td>
<td>---</td>
<td>Inactivation Kinetics</td>
</tr>
<tr>
<td>$\gamma_5$</td>
<td>CaG5</td>
<td>CNS</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$\gamma_6$</td>
<td>CaG6</td>
<td>CNS; Cardiac &amp; Skeletal Muscle</td>
<td>---</td>
<td>Attenuates $I_{\text{Ca}^\text{tac}}$ (Low-Threshold VGCCs)</td>
</tr>
<tr>
<td>$\gamma_7$</td>
<td>CaG7</td>
<td>CNS; Cardiac, Respiratory &amp; Reproductive Systems</td>
<td>---</td>
<td>Attenuates $I_{\text{Ca}^\text{tac}}$</td>
</tr>
<tr>
<td>$\gamma_8$</td>
<td>CaG8</td>
<td>CNS; Spinal Cord; Reproductive System</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$\alpha_2\delta-1$</td>
<td>CaA$_2.1$</td>
<td>CNS; Cardiac &amp; Skeletal Muscle</td>
<td>Domain III</td>
<td>Increase $I_{\text{Ca}^\text{tac}}$, Activation/inactivation, Trafficking of $\alpha_1$ to membrane, V-dependence of activation</td>
</tr>
<tr>
<td>$\alpha_2\delta-2$</td>
<td>CaA$_2.2$</td>
<td>CNS; Spinal Cord; Cardiac, Respiratory, Endocrine &amp; Reproductive Systems</td>
<td>---</td>
<td>Increase $I_{\text{Ca}^\text{tac}}$</td>
</tr>
<tr>
<td>$\alpha_2\delta-3$</td>
<td>CaA$_2.3$</td>
<td>CNS, Cardiac &amp; Skeletal Muscle</td>
<td>---</td>
<td>Increase $I_{\text{Ca}^\text{tac}}$, V-dependence of Activation, SS Inactivation</td>
</tr>
<tr>
<td>$\alpha_2\delta-4$</td>
<td>CaA$_2.4$</td>
<td>Skeletal Muscle; Cardiac, Digestive &amp; Endocrine</td>
<td>---</td>
<td>Increase $I_{\text{Ca}^\text{tac}}$</td>
</tr>
</tbody>
</table>

Table 1.2: Auxiliary Subunits of Voltage-Gated Calcium Channels & Their Modulatory Effects. Adapted from Hoffmann et al., 1994; Birnbaumer et al., 1998; Catterall, 2000; Arikkath & Campbell, 2003; Klugbauer et al., 2003.
β subunits are completely cytosolic in their localization and have five domains of which the two central domains have the highest homology within the family (Birnbaumer et al., 1998; Hanlon et al., 1999). At least three functional domains are predicted to reside within the β subunit including a postsynaptic density-95/disc large/zona occludens (PDZ) domain, a Src homology domain (SH3) and a guanylate kinase-like domain (Arikkath & Campbell, 2003) which reside on domains 1, 2, and 4 respectively. Recent crystal structure evidence suggests that these domains may actually interact with each other in forming the functional β subunit (Chen et al., 2004; Opatowsky et al., 2004; Opatowsky et al., 2004b). β subunits bind to α subunits through their β interacting domain (BID) and the AID on the α subunit (Pragnell et al., 1994; De Waard et al., 1994).

αδ subunits consist of both the α and δ portions, which are attached via a disulphide bond (Gurnett et al., 1996). The δ portion is an integral membrane protein with a single transmembrane region and a short intracellular C-terminus (Gurnett et al., 1996). The α portion is completely extracellular and highly glycosylated which appears to be integral for its effect on I, and its stable binding to the α subunit (Gurnett et al., 1996). Only the α portion of the subunit appears to interact extracellularly with the third transmembrane domain of the α subunit although other unknown interaction sites have also been postulated (Gurnett et al., 1997).

Finally γ subunits share a four subunit transmembrane domain, which is highly conserved within the family (Arikkath & Campbell, 2003). Neuronal forms of the γ subunit contain a target site for phosphorylation by cAMP and cyclic guanosine monophosphate (cGMP). Although it is known that the first half of the γ subunit is crucial for its interaction with the VGCC, it remains unknown exactly how this interaction takes place (Arikkath et al., 2003; Arikkath & Campbell, 2003). See Fig. 1.4 for a schematic of VGCC structure.

1.3.6 Localization and Expression of VGCCs within the CNS.

Focusing on the mammalian CNS, an interesting trend arises in terms of the types of VGCCs employed in mediating synaptic transmission. Despite their close biophysical properties, the localization of N and P/Q-type VGCCs may be segregated between neurons, across development, and even within AZs of the same neuron (Craig & Boudin, 2001; Reid et al., 2003).

N-type VGCCs appear to be responsible for a large portion of SV release early in development. Evidence for this appears from a number of preparations including hippocampus (Scholz & Miller, 1995), the calyx of Held (Iwasaki & Takahashi, 1998), cerebral cortex (Iwasaki et al., 2000; Millán et al., 2003), Cerebellum, Thalamus, Spinal Dorsal Horn (Iwasaki et al., 2000). During development, the contribution of N-type VGCCs declines in favour of P/Q-type mediated-release in all of these systems. This is particularly evident and well demonstrated at the calyx of Held (Iwasaki & Takahashi, 1998; Fedchyshyn & Wang, 2005). In general, R-type VGCCs play a minor role in mediating release throughout synaptic development but are thought to play a role in some forms of synaptic plasticity (Iwasaki & Takahashi, 1998; Dietrich et al., 2003; Reid et al., 2003).

A number of possible explanations have been proposed accounting for the differential expression and developmental switching of VGCC subtypes. Linkage to mGluRs (Scanziani et al.,
the distribution of Munc13 (Rosenmund et al., 2002), the size of terminals (Atwood & Karunanithi, 2002), and the coupling nature of VGCCs and the release machinery (Rozov et al., 2001) have all been identified as possible factors determining the placement of certain VGCC subtypes. The protein sequence for localization of VGCCs is thought to be located on the C-terminus of the α_1B subunit, which interacts with both Mint1 and CASK on N-type VGCCs (Maximov & Bezprozvanny, 2002). Alternatively, the α-neurexins may serve this function (Missler et al., 2003). Finally, the synaptic protein interaction site ("synprint") site, found on all N and P/Q-type VGCCs on the II-III linking region, may be involved in targeting to the AZ but does not explain the widespread differential targeting strategies employed throughout the CNS (Mochida et al., 2003a; Mochida et al., 2003b).

Ultimately, neither the mechanisms by which, nor the physiological reasons for alternative targeting of N and P/Q-type VGCCs is well understood (Reid et al., 2003). However, while their biophysical differences are subtle, modulation of N and P/Q-type VGCCs may occur through distinct pathways that compartmentalize signal transduction to a subset of VGCCs. The mechanisms of VGCC modulation will be discussed further below. Their sites of interaction on VGCCs are illustrated schematically in Figure 1.5.

### 1.3.7 Modulation of Presynaptic VGCCs – G-Protein Coupled Receptors.

As discussed above, VGCC activity is influenced by a number of G-protein-mediated regulatory pathways. mGluRs, GABA_βRs, A1Rs, and 5-HT receptors all signal their effect on VGCCs through G-proteins at the calyx of Held and, as discussed, do so to a greater or lesser extent depending on the developmental stage of the synapse.

G-protein-coupled receptors (GPCR) share many commonalities in structure and activation mechanisms. Structurally, all contain an extracellular N-terminus, intracellular C-terminus, seven transmembrane domains, three intracellular linking loops, and three extracellular linking loops (Tedford & Zamponi, 2006). Mechanistically, all GPCRs, when activated by agonists, facilitate exchange of Gα-GDP for GTP from the cytoplasm which then results in dissociation into Gα-GTP and Gβγ signalling components of which there are a variety of subtypes (Tedford & Zamponi, 2006).

Dunlap & Fischbach (1978, 1981) were the first to characterize the influence of these receptors on VGCCs, N-type in this particular case. Two signalling candidates have been proposed to account for GPCR inhibition of VGCCs being the Gα (i or o forms) through a diffusible pathway and Gβγ through a membrane-tethered or delimited pathway (Herlitze et al., 1996; Ikeda, 1996). Recent consensus is that the modulation of both P/Q and N-type VGCCs occurs through the membrane delimited pathway and Gβγ (Catterall, 2000; Tedford & Zamponi, 2006).

This type of inhibition acts on the VGCC to decrease whole-cell peak I_{Ca}, depolarize the voltage-dependence of activation, and slow the kinetics of activation and inactivation, which can be relieved by strong hyperpolarizing stimulus or high-frequency AP stimulation (Bean, 1989; Hille, 1994; Brody et al., 1997; Takahashi et al., 2000). The action of Gβγ on the VGCC was determined to arise from its stabilizing effects on the closed state of the VGCCs themselves, making them “reluctant” to open (Bean, 1989; Patil et al., 1996; Zamponi & Snutch, 1998). The transition between “reluctant” and “willing” states
Figure 1.5: Modulatory Protein Interaction Sites on a Generic $\alpha$, Subunit.

Schematic illustration showing approximate interaction sites for a number of proteins and modulatory factors as discussed in Chapter 1. Auxiliary subunits have been removed for clarity. Illustrated $\alpha$, subunit (blue) and GPCR (cyan) are simplified generic representations of high-threshold VGCCs and a number of GPCRs found at the calyx of Held-MNTB synapse. Upon activation of GPCRs and hydrolysis of GTP, Gbg subunits of the GPCR dissociate and act through a membrane-delimited pathway to modulate VGCCs at the sites shown. Adapted from Meir et al., 1999; Catterall, 2000; Arikkath & Campbell, 2003; Doering & Zamponi, 2003; Zamponi, 2003; Jarvis & Zamponi, 2005; Tedford & Zamponi, 2006; Lisman et al., 2007.
is believed to be due to the binding and unbinding respectively of the Gβγ subunit to the VGCC. Most evidence suggests that the site of Gβγ interaction is at the I-II linker (De Waard et al., 1997, Zamponi et al., 1997), however evidence also exists for roles of both the N- and C-termini in establishing the effect of Gβγ inhibition (Qin et al., 1997; Canti et al., 1999). It was demonstrated in later work that the binding of Gβγ at the C-terminus acts to primarily enhance the affinity of the VGCC for Gβγ. In addition, the N-terminus of the α1 subunit interacts with the I-II linker and, by unknown means, facilitates Gβγ binding (Li et al., 2004; Agler et al., 2005).

While the diverse means of activation of GPCRs have a well documented effect inhibiting VGCCs within the CNS, the diversity of these routes are not completely understood (Catterall, 2000; Evans & Zamponi, 2006; Tedford & Zamponi, 2006). At the calyx of Held it is intriguing to ask why the expression of many of these GPCRs is down-regulated with development (see above). Given the restrictive morphology of the immature calyx of Held terminal and the multiple GPCR pathways that converge to inhibit VGCCs, it is clear that there is a strategy to manage I_{Ca} more intensively at this age than in maturity. In addition, the more robust action of GPCRs on N-type VGCCs would further amplify the presence of these pathways at the immature calyx of Held. Indeed, G-protein inhibition of N-type VGCCs has been shown to completely restrict “reluctant” VGCCs from contributing to I_{Ca} evoked by single APs at chick ciliary ganglion neurons (Artim & Meriney, 2000). The purpose of these changes and their underlying mechanisms provide intriguing areas of future study at this synapse.

1.3.8 Modulation of Presynaptic VGCCs – PKC.

An additional source of VGCC modulation that has a more pronounced effect on N-type VGCCs is phosphorylation of the I-II linker by protein kinase C (PKC) (Stea et al., 1995). Activation of PKC has both direct and indirect affects on N-type VGCCs (Evans & Zamponi, 2006; Tedford & Zamponi, 2006). The direct affect of phosphorylation itself causes an upregulation of current activity (Stea et al., 1995). The indirect effect of PKC phosphorylation is to inhibit binding of the Gβγ subunit, as the phosphorylation site and Gβγ binding site are coincident on the I-II linker (Zamponi et al., 1997). Interestingly, action of PKC appears to affect only the Gβ1 subunit (Doering et al., 2004).

PKC phosphorylation also interferes with binding of syntaxin 1A at the “synprint” site, removing the inhibitory effects of this binding interaction also (Yokoyama et al., 2005). In this sense, N-type VGCCs may act as integration centers for a number of modulatory pathways, an ability that appears uniquely prominent in this subtype of VGCC within the CNS (Evans & Zamponi, 2006; Tedford & Zamponi, 2006).

1.3.9 Modulation of Presynaptic VGCCs – Ca^{2+}/Calmodulin/NCS-1.

P/Q-type VGCCs, but not N-type VGCCs, exhibit a unique form of Ca^{2+}-dependent facilitation in current amplitudes with continual stimulation at the calyx of Held (Cuttle et al., 1998; Borst & Sakmann, 1998). The mediation of this facilitation by P/Q-type VGCCs has been inferred from experiments performed in P/Q-type VGCC knockout (KO) mice, which do not exhibit this form of plasticity (Inchauspe et al., 2004; Ishikawa et al., 2005). P/Q-type Ca^{2+} current facilitation was believed to arise from an
interaction between calmodulin and the α₁ subunit of recombinant channels (Catterall, 2000; DeMaria et al., 2001). In these channels, calmodulin acts to accelerate gating kinetics, however, even with the acceleration mediated by calmodulin, the recombinant channels were 10 fold slower in their gating kinetics than those observed in intact nerve terminals (Borst et al., 1995; Sakaba & Neher, 2001; Takahashi, 2005b). Previous studies, performed at the calyx of Held, further eliminated relief from G-protein mediated inhibition as a potential signal for I_{Ca} facilitation but confirmed Ca^{2+} as underlying the phenomenon (Cuttle et al., 1998).

In 2002, Tsujimoto et al. identified neuronal Ca^{2+} sensor 1 (NCS-1) as the protein mediating I_{Ca} facilitation from P/Q-type VGCCs drawing question to the role of calmodulin in this process. Activation of NCS-1 causes increases in the activation kinetics, a hyperpolarizing shift in the maximal activation potential of VGCCs, but had little effect on the maximal plateau I_{Ca} suggesting that it’s effect does not alter single channel current (Tsujimoto et al., 2002). The mechanism through which Ca^{2+} binding to NCS-1 acts to facilitate P/Q-type VGCCs remains unknown.

Interestingly, this form of VGCC modulation acts specifically on the channels that are not modulated effectively by many of the previously discussed mechanisms (i.e. GPCRs). In addition, they are modulated to an opposite end, enhancing I_{Ca} and transmitter release. The prevalence of P/Q-type VGCCs with maturity and the amplifying effect of NCS-1 may serve to transiently maintain synaptic transmission during trains of APs by boosting I_{Ca} in an activity-dependent manner, a very different strategy to the many forms of inhibition targeted at N-type VGCCs in the immature synapse.

1.3.10 Modulation of Presynaptic VGCCs – Active Zone Proteins.

Both N- and P/Q-type VGCCs have been shown to associate with a variety of proteins (Catterall, 2000; Zamponi, 2003; Spafford & Zamponi, 2003; Tedford & Zamponi, 2006). Amongst the most interesting in the examination of presynaptic function are proteins of the AZ and the soluble NSF attachment receptor (SNARE) proteins. The first identified interaction site identified between N and P/Q-type VGCCs and the SNARE protein synaptotagmin was termed the “synprint” site and resides within the II-III linker (Sheng et al., 1994; Atlas et al., 2001). The hypothesized role of the interaction was to bring VGCCs and SVs into close proximity with each other ensuring fast synaptic transmission. However, binding of syntaxin 1 or synaptosome-associated protein of 25,000 daltons (SNAP-25) to VGCCs has also been shown to cause a hyperpolarizing shift in steady-state inactivation limiting the availability of VGCCs (Bezprozvanny et al., 1995; Zhong et al., 1999; see Spafford et al., 2003). These finding raised the possibility that proteins within the active zone not only functioned in a structure/function capacity but also as modulators of ion channels. However, in the presence of both syntaxin 1A and SNAP-25, N-type VGCC activity is restored suggesting that the proper functioning, channel open probability (P_{o}), and distribution of VGCCs depends on their localization with a “complete” release apparatus (Jarvis et al., 2002). P/Q-type VGCCs show similar characteristics but require the addition of synaptotagmin 1 to SNAP-25 and syntaxin 1A before normal channel activity is restored (Zhong et al., 1999). More recently, syntaxin 1A was shown to both inhibit VGCCs and serve to couple them to the release machinery in vivo suggesting a complex interaction for this protein at the AZ (Keith et al., 2007).
In an additional role, syntaxin 1A was found to bind to the G-protein β subunit and cause a tonic G-protein-mediated inhibition of VGCCs (Jarvis et al., 2000). The inhibition was voltage-dependent, independent of GPCR activation, and appears to be due to syntaxin 1A promoting the close colocalization of Gβγ with the VGCC (Jarvis et al., 2000). Interestingly, it appears as though activation of P/Q-type VGCCs can trigger the genetic expression of syntaxin 1A specifically, which would then act most potently to inhibit N-type VGCCs, providing an intriguing means of inter-VGCC regulation (Sutton et al., 1999; Spafford & Zamponi, 2003).

PKC and Ca²⁺/calmodulin kinase II (CaMKII) appears to be involved in this form of VGCC modulation also as PKC phosphorylation of the VGCC can prevent the binding of syntaxin 1A but cannot dislodge it once bound, further strengthening the role of PKC as a stimulant particularly for N-type VGCC activity (Yokoyama et al., 2005).

Through these diverse mechanisms of VGCC regulation, synapses of different developmental stages may employ differing strategies for the management of their SV output. In immature synapses, it appears that a combination of N-type VGCCs and a prevalence of GPCR expression provides a number of potent avenues through which to reduce N-type VGCC activity and minimize SV release. This may be an adaptation to reduce the incidence of postsynaptic receptor desensitization/saturation or to reduce the rate of exhaustion of a limited number of SVs within the immature RRP. In contrast, mature synapses have fewer means of VGCC modulation due to the prevalence of P/Q-type VGCCs and the limited effectiveness that GPCRs have on their activity (see above). NCS-1 appears to be one of the most potent modulators of P/Q-type VGCC activity, but does so through potentiation in contrast to the prevalence of attenuating signals in the immature synapse. Future investigation is required to determine the consequences of these distinct VGCC modulation strategies.

1.4 THE PRESYNAPTIC ACTIVE ZONE & SYNAPTIC VESICLE CYCLE

1.4.1 The Presynaptic Active Zone.

The presynaptic AZ is generally described as the location where SVs dock and fuse apposed to the postsynaptic density (PSD). The PSD contains various ionotropic receptors and their associated cytoskeletal and regulatory proteins and will not be discussed further within the scope of this thesis (for recent reviews see Boeckers, 2006; Okabe, 2007). Both the AZ and PSD are identifiable as electron dense regions, appearing “fuzzy” in electron micrographs (Schoch & Gundelfinger, 2006). While certain models of SV uptake (i.e. “kiss-and-run” & “kiss-and-stay”) would expand the definition of the AZ to include endocytosis, we will maintain the locations of these processes as distinct in discussion for simplicity.

The AZ contains a myriad of proteins involved largely in the various phases of the SV cycle including recruitment, docking, priming, fusion, and ultimately re-uptake (Südhof, 2004). Combined, these proteins make up the “cytomatrix” of the AZ (CAZ) (Dresbach et al., 2001; Harlow et al., 2001; Siskou et al., 2007). Although a largely simplified list, proteins comprising the cytoskeleton (actin,
tubulin, myosin, spectrin), scaffolding (CASK, Mint1, SAP-97, Velis, synapsins), multi-protein interactions (RIM, Rabs, ELKS, Piccolo, Bassoon, Munc-13), SV docking and fusion (SNAREs, Munc-18, NSF, α-SNAP), and cell adhesion (neurexins, cadherins, integrins, sidekicks) are all components of the CAZ (Schoch & Gundelfinger, 2006). The high density of proteins in the CAZ makes them appear as a web-like mass of interconnected filaments observable in high-resolution EM images (Bloom & Aghajanian, 1968; Landis et al., 1988; Harlow et al., 2001). Despite the potential complexity of regions with this number of interacting proteins, very few of the CAZ proteins affect the localization of others upon disruption or complete absence, suggesting a highly redundant, or distinct, means of establishing these proteins independently at the AZ (Schoch & Gundelfinger, 2006).

A schematic illustration of some of the key CAZ proteins, are shown in Figure 1.6. Amongst some of the most intensely studied CAZ proteins are the synapsins, although their exact function in the SV cycle remains unknown (Südhof, 2004; Schoch & Gundelfinger, 2006). Synapsins were originally hypothesized to anchor SVs to the actin cytoskeleton and perhaps aid in their transiting to the AZ (Roshal et al., 1995). Synapsins form filamentous strands and bind to both actin and SVs making them a good candidate for this role (Dresbach et al., 2001). However, genetic deletion of both synapsins I and II resulted in identical distribution patterns of SVs within the AZ but in reduced numbers (Roshal et al., 1995). In these mice, the rate of STD was increased, presumably due to more rapid depletion of the smaller RRP. These results suggest that synapsins play a role in the trafficking of SVs to the AZ however, they do not appear to perform this task exclusively. One interesting characteristic of the synapsins is that their interaction with both actin and SVs depends on their state of phosphorylation. When phosphorylated by CaMKII or myosin light-chain kinase (MLCK), synapsins dissociate from actin and SVs increasing their release and vice-versa when dephosphorylated (Bähler & Greengard, 1987; Greengard et al., 1993). Since proteins believed to be synapsins are not observed extending in close proximity to the AZ membrane, other proteins must facilitate the movement of SVs to the AZ where release machinery engagement can occur (Lin & Scheller, 2000; Dresbach et al., 2001; Südhof, 2004; Schoch & Gundelfinger, 2006).

Rab3-interacting molecules (RIM), and their namesake Rab3 proteins, appear to serve as intermediary interacting proteins with binding partners spanning a number of CAZ proteins. RIMs interact with both Munc-13 and Rab3 at its N-terminus but at distinct sites. In the absence of RIM1α, priming of SVs is reduced by ~50% while also reducing the expression of Munc-13 by a similar amount (Schoch et al., 2002; Calakos et al., 2004). These data, and the identified binding partners for RIM, suggest that its function is to aid in transiting SVs from their anchor at the actin cytoskeleton to the AZ membrane (Dulubova et al., 2005; Betz et al., 2001). In addition, RIM binds to ELKS at its central PDZ domain (Ohtsuka et al., 2002) and to α-Liprin on its C-terminal C2B domain (Schoch et al., 2002). However, due to its diverse binding partners and range of apparent functions at different synapses (i.e. LTP), establishing an exact role for RIMs is difficult (Schoch & Gundelfinger, 2006).

As discussed above, ELKS (i.e. CAZ-associated protein or CAST) binds to RIM1α and functions to aid it in transiting SVs to the AZ, but ELKS also interact with piccolo and bassoon (Ohtsuka et al.,
Figure 1.6: The Cytomatirx of the Presynaptic Active Zone.
Simplified schematic illustration of the components that make up the cytomatrix of the presynaptic active zone. Specific interaction partners and functional roles of these components are outlined within Chapter 1. Adapted from Gundelfinger & tom Dieck, 2000; Dresbach et al., 2001; Schoch & Gundelfinger, 2006.
Liprin-α has also been shown to bind to ELKS as well as RIM and piccolo (Kim et al., 2003; Schoch et al., 2002). These interactions are not well understood but appear to be required for normal synaptic transmission (Takao-Rikitsu et al., 2004).

Munc-13 is crucial for the maturation and priming of SVs and their acquisition of fusion competency as illustrated in Munc-13 KO studies (Augustin et al., 1999). Munc-13 has a diverse list of binding partners including RIM1α and calmodulin at its N-terminus (Betz et al., 2001; Junge et al., 2004) and syntaxin at its C-terminus (Betz et al., 1997; Basu et al., 2005) amongst others. By determining the fusion competency of SVs, Munc-13 determines the size of the RRP, which has a significant role in determining the output capacity of the synapse (Fernández-Alfonso & Ryan, 2006). Interestingly, the variety of Munc-13 expressed in an individual neuron appears to determine P_{BR} to the extent that synapses exhibit either STD or STF in conjunction with the Munc-13 subtype (Junge et al., 2004).

Piccolo and bassoon are some of the largest presynaptic proteins identified and are structurally related to each other (tom Dieck et al., 1998; Fenster et al., 2000). Interestingly, these proteins are found only in vertebrates and are thought to extend upward from the AZ membrane to contact the SVs in the periphery. Piccolo can interact with vesicle-associated membrane protein (VAMP) indirectly through Rab3 and appears to provide a link between SV-related proteins and the AZ through its binding to L-type VGCCs (Fujimoto et al., 2002). Furthermore, the Q-domain of piccolo has been shown to interfere with clathrin-mediated endocytosis suggesting that it is a protein which links many of the processes occurring at the presynaptic AZ membrane (Fenster et al., 2003). Bassoon is important in the assembly and morphological development of synapses as its absence results in morphological abnormalities in some AZs (Dick et al., 2003; Dresbach et al., 2001).

Amongst the array of presynaptic proteins, those that interact with VGCCs are of particular interest as candidates for establishing AZ geometry. Localization of VGCCs within the AZ is of particular importance as their tight coupling to SVs is crucial for synaptic transmission on the timescales of that observed in nature. This necessity has provoked hypotheses of anchoring proteins that retain VGCCs in close proximity to the SV release machinery. The detailed images of Harlow et al. (2001) show that there appears to be such a physical linkage between VGCCs and SV built into the AZ of the frog neuromuscular junction. At the AZ, the membrane associate guanylate kinases (MAGUK) family of proteins, including CASK, forms a link between cell adhesion molecules in the presynaptic terminal like β-neurexin (Hata et al., 1996) and the PSD. Other modular adapter proteins such as Mint1, along with CASK, also bind to the long C-terminal splice variants of N and P/Q-type VGCCs and are thought to localize them to the AZ (Butz et al., 1998; Maximov et al., 1999; Maximov & Bezprozvanny, 2002). These proteins provide some of the best current candidates for proteins that tie VGCCs to the AZ. An alternative hypothesis is that VGCCs bind directly to the release machinery proteins themselves through the “synprint” site located on the II-III linker. The synprint site has been shown to bind to syntaxin1, SNAP-25, synaptotagmin1, and cysteine string protein (CSP), through it is not clear whether this interaction site is necessary for the appropriate localization of VGCCs at the AZ (Mochida et al., 2003; Spafford & Zamponi, 2003; Jarvis & Zamponi, 2005). In *Lymnaea* neurons, the VGCCs mediating SV release do not contain a synprint site and do not show any binding to release machinery proteins.
suggesting that this interaction is not crucial for the placement of VGCCs. In contrast, these VGCC do show interaction domains with Mint1 and CASK (Spafford & Zamponi, 2003; Spafford et al., 2003). Furthermore, the synprint region differs between N and P/Q-type VGCCs (Sheng et al., 1998) and binds to such a large number of proteins (Seagar et al., 1999) that deducing its importance in linking these fundamental components of SV release is difficult. The binding of synaptotagmin to the synprint site of both N and P/Q-type VGCCs has been shown to prevent the interaction between synaptotagmin and syntaxin suggesting it may be crucial for the initiation of exocytosis (Sheng et al., 1997).

Ultimately, the array of presynaptic proteins stretches beyond those components listed here, with elucidation of their particular function being particularly difficult due to the web of interactions, both direct and indirect, between them (Südhof, 2004; Dresbach et al., 2001; Schoch & Gundelfinger, 2006). While these proteins can all be localized to the CAZ, future investigation must focus on their exact function and the numerous ancillary effects that manipulation of any of these proteins may have on the system as a whole.

### 1.4.2 Synaptic Vesicle Recruitment & Docking.

The SV cycle begins with the transiting of SV from the periphery, which can be either the reserve pool of SVs (RVP) or the regions of endocytosis, to the AZ (Fig. 1.7A) (Südhof, 2004). The preceding steps involving filling of the SVs with transmitter will not be discussed further here. Myosin motors are believed to transit SVs along actin filaments seen projecting away from the AZ (Bridgman, 1999). Rab3 and synapsins have been hypothesized to link the SVs to the actin cytoskeleton either directly or indirectly (Lin & Scheller, 2000; Dresbach et al., 2001). It is the cycling ability of Rab3 between a GTP-bound state that associates with SVs and a GDP-bound state that dissociates with SVs, and the phosphorylation-dependent interactions of synapsins with SVs (as above), which make them strong candidates for the protein link at this step (Bähler & Greengard, 1987; Greengard et al., 1993; Lin & Scheller, 2000). It is at the completion of the actin-myosin chain at which one of these proteins may act to free the SV and thus expose VAMP for its exocytotic role (Prekeris & Terrian, 1997).

Following arrival at the CAZ, SVs are passed to a yet unconfirmed secondary transit system that brings them in close apposition to the membrane-bound components of the SNARE complex. Currently, in vertebrates, piccolo and bassoon are good candidates for this role as their large size and diverse protein binding sites provide ample room for interaction with SV-bound proteins (Dresbach et al., 2001; Südhof, 2004). Piccolo interacts with SVs most likely through VAMP and Rab3 and acts to bring them closer to the membrane. In addition, piccolo could interact with RIM or RIM-binding proteins (RIM-BP) to serve a similar function. The termination of this step appears to require Munc-13 which may be involved in establishing the final placement of the vesicular SNARES (v-SNARES) and target SNARES (t-SNARES) at the AZ membrane (Augustin et al., 1999; Dresbach et al., 2001). Indeed, in the absence of Munc-13, this stage of the SV cycle does not occur (Augustin et al., 1999). While the exact sequence of steps in passing SVs from the actin cytoskeleton to the t-SNARES is unknown, the above key proteins appear to be important in the process.
Figure 1.7: The Synaptic Vesicle Cycle.

(A) Schematic illustration of the synaptic vesicle cycle. SVs begin as part of the RVP, are mobilized to the AZ, and align with release sites as part of the docking/priming phase. Upon Ca$^{++}$ entry into the terminal and binding to synaptotagmin, conformational changes in the SNARE complex result in fusion of the SV and AZ membranes. SVs are then recycled either at the AZ, termed “Kiss-and-Run”, or through endosomal and clathrin-mediated endocytosis. The newly recycled SVs are re-acidified and re-filled with neurotransmitter for subsequent rounds of the cycle. (B) Magnification of the boxed region in A showing the interaction of SNARE proteins and the fusion of the SV and AZ membranes. Details of the illustrated processes are given in Chapter 1. Adapted from Lin & Scheller, 2000; Dresbach et al., 2001; Südhof, 2004; Lisman et al., 2007.
Docking of SVs however, is better understood than the processes above. Initially, docking involves NEM-sensitive factor (NSF) and potentially the septin family of proteins (see below). NSF binds to the SNARE complex via a cytosolic protein, α-SNAP, forming a 20S complex. Hydrolysis of ATP by NSF breaks the 20S complex into its components (7S), which is the fusion competent state of the t-SNAREs (Lin & Scheller, 2000). Recent investigation suggests that the action of NSF may serve to exclude other fusion inhibiting proteins from the release machinery making its role in SV priming crucial. In addition, release competence of the t-SNAREs requires the conversion of syntaxin, from the closed conformation, into the open form, which is capable of participating in the docking-competent ternary SNARE complex. The closed conformation is stabilized by Munc-18 (Dulubova et al., 1999), an interaction which is broken when Rab interacts with Munc-18, leaving syntaxin in the open conformation and capable of binding the v-SNAREs (Lin & Scheller, 2000). Some evidence suggests that synaptophysin may perform a similar role at the SV by determining VAMP receptiveness for the t-SNARE complex (Edelmann et al., 1995).

With the delivery of a SV in close proximity to the AZ and the conversion of the t-SNARE complex into a receptive state, the process of SV fusion can occur upon the detection of Ca\(^{2+}\) influx. Linking SVs in the periphery to the AZ appears to occur through Rab3-piccolo-RIM and/or Munc-13-synatxin interactions and is a key target for future mechanistic clarification (Dresbach et al., 2001).

1.4.3 SNARE Assembly & Synaptic Vesicle Fusion.

It is generally accepted that the fusion of SVs requires the interaction of SNARE proteins. Three SNAREs comprise the minimal machinery necessary for coordinated synchronous SV release to occur; VAMP, syntaxin 1, and SNAP-25 (Söllner et al., 1993; Südhof, 2004). This core complex is formed with the H3 domain of syntaxin 1, the lone coiled-coil domain of VAMP, and both coiled-coil domains of SNAP-25 form an intertwined ternary complex (Sutton et al., 1998). The formation of this complex establishes the complete docking of the SV at the release site with its “zippering” encouraging fusion of the SV and AZ membranes (Lin & Scheller, 2000; Südhof, 2004; Lisman et al., 2007). Interestingly, while the SNAREs of the ternary complex are crucial for synchronous release of SVs, KO studies of VAMP and SNAP-25 show that asynchronous and spontaneous release of SVs still occurs in their absence. In these synapses, a small amount of stimulated release can also be triggered (Verhage et al., 2000; Schoch et al., 2001; Washbourne et al., 2002), suggesting that there may be some redundancy in SNARE function or other means of triggering SV release.

Once the ternary complex is formed, it is believed that SVs in this arrangement form unstable hemipores with the AZ membrane (Jahn et al., 2003; Südhof, 2004; Reese et al., 2005; Xu et al., 2005; Zampighi et al., 2006; Lisman et al., 2007) and that complexin proteins aid in its stabilization. This configuration then waits for a Ca\(^{2+}\) signal to trigger the final steps of full SV fusion.

Upon Ca\(^{2+}\) entry at the AZ, domains of high-concentration are created in the vicinity of the VGCC pore. Ca\(^{2+}\) diffuses away and contacts Ca\(^{2+}\)-binding proteins that serve a number of functions (signalling, buffering etc…). Synaptotagmin 1 and 2 are generally accepted as the Ca\(^{2+}\)-binding proteins that trigger the fast synchronous exocytosis of SVs. Both of these Ca\(^{2+}\) sensors are very similar in structure and
function with the key difference that synaptotagmin 1 has a 2-fold higher affinity for Ca\(^{2+}\) than does synaptotagmin 2 (Sugita et al., 2002). Synaptotagmin 1 and 2 bind five Ca\(^{2+}\) ions, three at the C\(_{\alpha}\)A domain and two at the C\(_{\beta}\)B domain (Ubach et al., 1998; Fernandez et al., 2001), which agrees with current modelling studies requiring 5 binding sites at the calyx of Held (Bollmann et al., 2000; Schneggenburger & Neher, 2000) and the seminal studies on the subject (Dodge & Rahamimoff, 1967). Binding of Ca\(^{2+}\) ions to the five sites is cooperative, meaning that the affinity for each binding site is increased after Ca\(^{2+}\) binding to the previous site although the mechanism for this is not well understood (Lisman et al., 2007). Current models hypothesize that Ca\(^{2+}\) binding to synaptotagmin causes a conformational change in the molecule that encourages the “zippering” of the SNARES and the interaction of the C2 domains with phospholipids of the AZ membrane (Sugita et al., 2002; Südhof, 2004). These interactions pull the SV closer to the AZ and supply the necessary energy to promote full fusion of the membranes and the release of SV contents into the synaptic cleft (Fig. 1.7B).

There is some debate surrounding a number of aspects of the fusion pore. First, it is unknown whether the pore is lined by lipids or proteins (Han et al., 2004; Jackson & Chapman, 2006; Lisman et al., 2007). Second, it is a matter of debate whether, and to what extent, the pore develops (i.e. narrow, wide, transient or full fusion etc…). While these particulars of SV fusion provide interesting targets of study, they are beyond the scope of the topics discussed herein. Due to the small size of SVs and the extreme concentration gradient that exists between the extracellular space and the interior of the SV, we will assume that, in all but very rare instances, fusion of a SV by any means, results in the complete excretion of transmitter from the SV (see Südhof, 2004; Fernández-Alfonso & Ryan, 2006; He et al., 2006; Lisman et al., 2007).

### 1.4.4 Synaptic Vesicle Endocytosis.

The uptake of SVs ultimately determines the upper limit for output of the synapse as it refills the pool of depleted SVs (Fernandez-Alfonso & Ryan, 2006). There are three main hypotheses that describe the uptake of SVs once they have fused with the AZ membrane; the classical mechanism of clathrin-mediated endocytosis (Heuser & Reese, 1973), “kiss-and-run” (Ceccarelli et al., 1973) and “kiss-and-stay” (Barker et al., 1972), and bulk endocytosis (Koenig & Ikeda, 1996) (Fig. 1.7A). These mechanisms are first sorted by the location at which they occur, with both “kiss” mechanisms occurring without clathrin-mediated endocytosis close to the release site, and clathrin-mediated endocytosis occurring in the perisynaptic zone outside of the AZ (Südhof, 2004). Further separating the “kiss” mechanisms, ‘kiss-and-run” refers to SVs which undock and recycle locally without full fusion of the SV while “kiss-and-stay” refers to SVs which form a fusion pore but do not undock from the release apparatus. While it is generally accepted that the ‘kiss’ mechanisms do occur in the CNS, it is unknown to what extent these means of fusion pore establishment and SV recycling contribute to the overall pool of SV. One hypothesis is that they may serve to establish mechanisms with distinct kinetics with which SVs may be recycled depending on synaptic requirements (see He & Wu, 2007; Lisman et al., 2007). At the calyx of Held, direct recording from the release face provides evidence that a small portion of release is mediated by a “kiss” mechanism, the difference between “run” and “stay”, as defined here, being
indistinguishable using this approach (He et al., 2006). While the stimulation paradigm was non-physiological in this study, it suggests that full fusion of SVs, and most likely clathrin-mediated endocytosis, serves as the primary mechanism for SV fusion and uptake at this synapse.

It has been proposed that during periods of minimal SV use, “kiss” mechanisms are used to rapidly refill the few SVs used during spontaneous activity, whereas bulk endocytosis (Wu & Wu, 2007) using the slower clathrin-mediated pathway is employed due to its higher retrieval capacity (Südhof, 2004; Kavalali, 2006; Lisman et al., 2007). Evidence from the calyx of Held suggests that there is a fast and slow component to endocytosis that may reflect these two processes (Sakaba & Neher, 2001). Combined with the evidence from He et al. (2006), this may provide evidence to support the activity-dependent use of these distinct mechanisms of endocytosis (de Lange et al., 2003; Wu et al., 2005).

In clathrin-mediated endocytosis, adaptor protein 2 (AP2) is recruited to the plasma membrane through a potential interaction with synaptotagmin (Schmid, 1997), where it assembles clathrin into a cage surrounding the section of membrane targeted for endocytosis. This cage pulls the membrane into the presynaptic terminal where dynamins, with the addition of GTP, complex to form helices which pinch together the neck of the SV (Sweitzer & Hinshaw, 1998). A number of proteins aid in this process; the endophilins are hypothesized to aid in the formation of negative curvature of invaginated SVs (Schmidt et al., 1999) and amphipysins aid in the aggregation of dynamin or the fragmenting of the lipids at the endocytosis site (Takei et al., 1999). Once the SV has been separated from the presynaptic membrane, dynamin interacts with profilin and actin/dynamin binding protein 1 (Abp1). These interact with G and F-actin respectively, providing a link to the actin cytoskeleton and a potential link back into the releasable pool of SVs. In addition, the path of these new SVs may or may not go through an endosomal step on the way back to the releasable pool (see Kavalali, 2006) (Fig. 1.7A).

To support the rates of SV release observed at the calyx of Held, endocytosis must occur at sufficient rates and at a sufficient capacity to replenish the relatively large buffer of SVs at the AZ. While it is not yet clear which mechanisms are responsible under particular conditions of activity at this synapse, existing evidence supporting “kiss”, clathrin-mediated, and bulk retrieval of membrane suggests the prevalence of each may be regulated depending on the transient requirements of the synapse.

1.4.5 The Role of Septin Proteins in Synaptic Vesicle Release.

In addition to the partial list of presynaptic proteins and interacting partners given above, we have investigated a unique group of proteins originally identified as being crucial for the cytokinesis of budding yeasts called the septins (see Chapter 5) (Hartwell, 1971; Haarer & Pringle, 1987). Since their discovery, a number of septin proteins have been found in the mammalian brain, some with specific presynaptic localization (Kinoshita et al., 2000).

Septin 5 (Sept5) is a filamentous protein that is found associated with SVs and AZs through its interaction with syntaxin (Beites et al., 1999; Beites et al., 2005). This interaction is thought to prevent SV docking and association with t-SNARES by either spanning across the AZ or spanning between the AZ and SVs (Fig. 1.8). In addition, Sept5 may link SVs together by binding to 7S complexes between
Figure 1.8: The Predicted Role of Septin Proteins in Modulating Exocytosis.
Schematic illustration of the hypothesized action of Septin filaments in inhibiting SV exocytosis. Sept5 has been shown to bind competitively with α-SNAP to syntaxin which may underlie an interaction which allows for SV docking and priming. In this scheme, Sept5 binds to syntaxin either across the release site or between the release site and the SV. α-SNAP then competes-off Sept5 and allows NSF to bind to the SNARES forming a 20S complex. Upon ATP hydrolysis by NSF, the 20S complex breaks apart forming the 7S complex which is capable of accepting a SV. In this sense, Sept5 acts as a barrier for SV fusion and may prevent SVs from approaching fusion sites at which the SNARES remain in their closed conformation. Adapted from Beites et al., 2005.
SVs (Beites et al., 1999; Beites et al., 2005). Sept5 is able to interact with syntaxin 1A, at either end of its length, alone or as part of the 7S complex, at the same location as its other binding partners SNAP-25 and VAMP. However, it is unable to bind syntaxin in the presence of α-SNAP (Beites et al., 2005). Thus, Sept5 may act as a molecular “brake” preventing the association of the SNARE complex in the absence of α-SNAP. Septin filaments form in lengths of 25nm, the same approximate distance that SVs have been observed to come to rest, prior to fusion, at the goldfish retinal bipolar cell (Hsu et al., 1998). In addition, filaments of approximately 30nm, believed to be synapsins, have been visualized connecting SVs to each other and to the actin cytoskeleton (Hirokawa et al., 1989; Dousseau & Augustine, 2000). If these filaments were comprised of septins, at least in part, it could suggest that they play an additional role in the transiting and/or stabilization of SVs in the periphery. The difficulty in interpreting data from synapsin KO neurons makes the potential action of Sept5 an intriguing alternate hypothesis (see above).

1.5 CALCIUM & SYNAPTIC VESICLE RELEASE

1.5.1 Calcium Domains & Calcium Buffers.

In central synapses the release of SVs is triggered by Ca\(^{2+}\) ions binding to synaptotagmin (see above). Whether the Ca\(^{2+}\) signal is sufficient to engage this process depends on a number of factors that shape the spread of Ca\(^{2+}\) ions within the AZ. These processes can be generally separated into two types; those that determine the magnitude of Ca\(^{2+}\) influx at a given release site, and those that determine and modulate its migration within the terminal. The effects of the later are significantly impacted by the nature of the former (Simon & Llinas, 1985; Neher, 1998; Meinrenken et al., 2002).

While the opening of any ion channel is a probabilistic event, the factors that determine the open probability are well understood for VGCCs. The shape of the presynaptic AP ultimately determines Ca\(^{2+}\) flux into a release site. Upon depolarization, VGCCs open allowing Ca\(^{2+}\) ions to flow down their concentration gradient into the terminal and into the vicinity of SVs. Very rapidly (<1μs), a standing hemispherical “domain” of elevated [Ca\(^{2+}\)] is established which decays rapidly with distance from the channel pore (decreases with 1/r) (Klingauf & Neher; 1997; Naraghi & Neher, 1997; Neher, 1998; Meinrenken et al., 2002; Oheim et al., 2006). At a single VGCC these [Ca\(^{2+}\)] domains are termed “nanodomains”, the size of which is initially determined by the magnitude of Ca\(^{2+}\) flux through the nearly point source open VGCC (\(i_{Ca}\)). Large APs, near the reversal potential for \(i_{Ca}\) (~+30mV), increase the open probability and driving force for Ca\(^{2+}\) ions increasing \(i_{Ca}\), while wide APs increase the open probability and open time of VGCCs also increasing \(i_{Ca}\). In addition, modulation of VGCCs as discussed above (i.e. G-Proteins, auxiliary subunits, etc…) can shift the open probability, activation kinetics, and/or conductance of VGCCs. Any of these factors can determine the time-course and magnitude of Ca\(^{2+}\) influx through an individual VGCC (Augustine & Neher, 1992).

Once inside the terminal, the nature of the Ca\(^{2+}\) influx determines to what extent it will be affected by endogenous/exogenous buffering molecules. These may be both fixed and mobile, and determine how rapidly Ca\(^{2+}\) domains will spread and reach the Ca\(^{2+}\) sensor (Neher, 1998; Oheim et al.,
2006). Understanding the complex interaction between Ca\(^{2+}\) buffers and Ca\(^{2+}\) ions with fine detail requires the evaluation of a large number of parameters, many of which remain unknown in most preparations (Neher, 1998). However, some of the observed synaptic characteristics in accessible systems like the calyx of Held terminal, and use of secretory systems like chromaffin cells (Neher, 2006), have provided limits for some parameters. The extremely rapid kinetics of SV fusion upon Ca\(^{2+}\) entry, and the rapid fall-off in [Ca\(^{2+}\)] elevation with distance from the channel pore, implies that the Ca\(^{2+}\) sensor must be of relatively high affinity and must be located in close proximity to the Ca\(^{2+}\) source (Simon & Llinas, 1985; Stanley, 1986; Meinrenken et al., 2002). However, the exact nature of the iB\(\text{Ca}_B\) remains only partially understood due to the unknown number of VGCCs which cooperate in creating a suprathreshold [Ca\(^{2+}\)] signal (discussed below) (Meinrenken et al., 2002). In addition, complicating factors like other synaptic structures, which limit the spatial movement of Ca\(^{2+}\) ions within the terminal, further complicate a simple model of how Ca\(^{2+}\) ions disperse within the presynaptic cytosol (Shahrezaei & Delaney, 2004).

Here, in order to summarize the role of Ca\(^{2+}\) buffering in shaping [Ca\(^{2+}\)] domains, we will assume a simplified and idealized condition where Ca\(^{2+}\) is impacted only by buffers while in transit to the Ca\(^{2+}\) sensor (assumed to be synaptotagmin) (see also Rukasov, 2006). In addition, we will only discuss the effect of Ca\(^{2+}\) buffers in the steady state condition, where [Ca\(^{2+}\)] profiles have stabilized around the mouth of an open VGCC, which occurs on the order of μs (Naraghi & Neher, 1997).

Within the terminal, Ca\(^{2+}\) ions move only by passive diffusion, the rate of which depends on the diffusion constant of the medium, the concentration gradient across which the substance is diffusing, and temperature (Meinrenken et al., 2002; Oheim et al., 2006). The key function of Ca\(^{2+}\) buffers is to compartmentalize the spatial and temporal reach of a [Ca\(^{2+}\)] nanodomain such that its action is restricted to a given functional region (Roberts, 1994; Naraghi & Neher, 1997). This action depends on the composition and character of buffers, both endogenous and exogenous, within a given terminal. In particular, this depends on mobile buffers which themselves can be replenished by diffusion. Immobile Ca\(^{2+}\) buffers are saturated rapidly upon VGCC opening and generally serve to lengthen the time required for equilibrium of Ca\(^{2+}\) domain formation (Stern, 1992; Naraghi & Neher, 1997). Even the time that a [Ca\(^{2+}\)] nanodomain persists, before diffusing away, is shortened by the presence of Ca\(^{2+}\) buffers.

As Ca\(^{2+}\) ions bind to buffer molecules in the periphery of the domain, their effective concentration gradient is increased, encouraging more rapid diffusion from the highest concentration regions in the vicinity of the channel pore (Naraghi & Neher, 1997; Neher, 1998; Oheim et al., 2006). Ca\(^{2+}\)-bound buffers will effectively hand-off Ca\(^{2+}\) ions to other slower buffers which eventually pass Ca\(^{2+}\) to extrusion mechanisms (not discussed here) (Neher, 1998; Oheim et al., 2006). In this manner, Ca\(^{2+}\) buffers result in the rapid dissipation of Ca\(^{2+}\) nanodomains following the closure of a VGCC, rising and falling within microseconds (Neher, 1998). The rate of the rise and fall of Ca\(^{2+}\) nanodomains depends critically on the distance from the VGCC pore as buffer effectiveness changes dramatically with increasing distance from the Ca\(^{2+}\) source (Yamada & Zucker, 1992). This is directly related to the buffer “length constant”, often termed λ (Naraghi & Neher, 1997). This elegant means of characterizing a Ca\(^{2+}\) buffer uses a length dimension, dependent on the buffer properties, to describe regimes in which a given Ca\(^{2+}\) buffer will have very different effects on the spatiotemporal profile of [Ca\(^{2+}\)] (Naraghi & Neher, 1997; Neher, 1998). The
length constant can be defined as the average distance a Ca\textsuperscript{2+} ion will diffuse before being captured by the buffer and is determined by the dissociation constant, total buffer concentration, and on-rate of Ca\textsuperscript{2+} binding (Naraghi & Neher, 1997). Interestingly, the length constant defines two domains in which the interaction of the buffer and Ca\textsuperscript{2+} occur under differing influences. For distances longer than $\lambda$, the [Ca\textsuperscript{2+}] nanodomain is defined by the binding ratio and diffusion properties of the buffer (i.e. equilibrium properties). That is, when the diffusion time for Ca\textsuperscript{2+} is large compared with the on-rate of the buffer, the interaction of Ca\textsuperscript{2+} and the buffer is always at equilibrium and thus the decay of a [Ca\textsuperscript{2+}] nanodomain with distance from the VGCC will be modulated by those parameters (Naraghi & Neher, 1997). Conversely, at distances shorter than $\lambda$, Ca\textsuperscript{2+} ions diffuse away more rapidly than the buffer is capable of binding them. In this domain, Ca\textsuperscript{2+} is essentially unbuffered and decays with $1/r$ from the source (Simon & Llinas, 1985; Naraghi & Neher, 1997). Distances comparable to $\lambda$ are the locations in space where Ca\textsuperscript{2+} ions are transferred, either from the unbuffered domain to the buffer-bound state, or from the bound-state of one buffer to that of another. In the presence of multiple buffers, the individual $\lambda$ of each buffer will be the point where Ca\textsuperscript{2+} ions are transitioned from one buffer to the next. Interestingly, the larger a buffers $\lambda$, the smaller the buffer saturation and the larger distance over which the buffer will be highly effective at binding Ca\textsuperscript{2+}. That is, fast buffers with short $\lambda$ are effective at binding Ca\textsuperscript{2+} only over a short distance range from the VGCC pore and are prone to saturation. They must rely on other buffers with longer $\lambda$ to take up their Ca\textsuperscript{2+} ions to curtail saturation (Naraghi & Neher, 1997). Within each of these buffering shells, the volume between the different buffer $\lambda$’s, the dissipation of Ca\textsuperscript{2+} ions will be impacted primarily by the buffer with a shorter $\lambda$.

The simplifications above, which allow for the conceptual rationalization of buffer effects, are based on assumptions that may not be valid for all conditions. The linearization of buffered diffusion depends on the condition that Ca\textsuperscript{2+} influx generates only small saturation levels (~20%) of the buffer, or that the buffer is in such excess that relative saturation is again small. In addition, this model does not account for long distances from the VGCC at which extrusion mechanisms may begin to affect the [Ca\textsuperscript{2+}] nanodomain (Naraghi & Neher, 1997; Neher, 1998; Oheim et al., 2006). However, for single VGCC currents, these assumptions appear to be valid. In addition, the superposition of such models may be extended to describe different arrangements of multiple VGCCs and their resultant [Ca\textsuperscript{2+}] at a certain point in space (Meinrenken et al., 2002). Around single VGCCs, the composition of Ca\textsuperscript{2+} buffers will determine the nature of the Ca\textsuperscript{2+} nanodomain, and compartmentalize it to a region defined largely by their length constants in the absence of significant saturation. This compartmentalization significantly influences the rationalization of the minimal release apparatus, the kinetics of the SV release process, the mechanisms of Ca\textsuperscript{2+}-dependent short-term plasticity, and even the expected sensitivity of the Ca\textsuperscript{2+} sensor (Simon & Llinas, 1985; Neher, 1998; Meinrenken et al., 2002; Meinrenken et al., 2003).

Indeed, the modelling of Ca\textsuperscript{2+} domains and buffered diffusion of Ca\textsuperscript{2+} has impacted the interpretation of many aspects of synaptic transmission. The rapid dissipation of [Ca\textsuperscript{2+}] domains with increasing distance from the VGCC pore called into question the classical residual Ca\textsuperscript{2+} hypothesis of STF (Yamada & Zucker, 1992). The discrepancy arose from the observation that STF persists for hundreds of milliseconds following an initial Ca\textsuperscript{2+} influx while the actual [Ca\textsuperscript{2+}] domain collapses orders of
magnitude more rapidly (~200μs). This prompted Yamada & Zucker (1992) to propose a facilitation sensor, distinct from the Ca$^{2+}$ sensor for release, which could detect the very slight [Ca$^{2+}$] increases that persisted away from VGCCs following activation. Secondly, the preliminary reports regarding the sensitivity of the Ca$^{2+}$ sensor, which were later found to be largely underestimated (~100μM vs. 1μM), forced interpretation as to the number and arrangement of VGCCs that would be required to trigger release of a SV (Simon & Llinas, 1985; Yamada & Zucker, 1992; Bollmann et al., 2000; Schneggenburger & Neher, 2000; Meinrenken et al., 2002). Based on experiments conducted with the exogenous Ca$^{2+}$ chelators EGTA and BAPTA, it was concluded that, at the calyx of Held, a single VGCC was incapable of triggering SV release and reproducing existing observations (Borst & Sakmann, 1996; Naraghi & Neher, 1997). However, in other systems it was hypothesized and demonstrated that a single SV and VGCC was capable of triggering release (Simon & Llinas, 1985; Stanley, 1996). These contradicting results sparked a long-standing, and currently ongoing, debate as to the number of VGCCs that are required for triggering SV release or “release modality”.

1.5.2 Molecular Cooperativity & Channel Cooperativity.

Release modality can be generally separated into two types; the “nanodomain” modality, which uses the Ca$^{2+}$ flux from a single tightly-coupled VGCC to trigger SV release, and the “microdomain” modality, which uses the combined influx from multiple VGCCs to trigger the release of a more loosely-coupled SV. The nature of Ca$^{2+}$ influx and buffering has been described above for the nanodomain modality, however the microdomain modality is more complicated to interpret. Considerations of VGCC topography (Meinrenken et al., 2002), and validation of current model assumptions such as small buffer saturation (Naraghi & Neher, 1997), must all be re-evaluated in the context of multiple Ca$^{2+}$ sources and the resultant complexity of the [Ca$^{2+}$] profile (Neher, 1998). For example, a cluster of VGCCs may produce a [Ca$^{2+}$] microdomain that saturates fast mobile buffers to the extent that the “unbuffered” domain extends to the λ of the next Ca$^{2+}$ buffer in line, thereby producing a larger unbuffered region of high [Ca$^{2+}$] than would be predicted in the nanodomain arrangement. Alternatively, with an incorrect estimation of Ca$^{2+}$ sensitivity, the number of contributing VGCCs required for SV release may be greatly exaggerated and highly variable (i.e. 60 VGCCs: Borst & Sakmann, 1996 vs. 12 VGCCs: Meinrenken et al., 2002). In addition, the arrangement of VGCCs and SVs has implications on the predicted STP, kinetics and dynamic range of the synapses (see Chapter 6 & 7). So while modelling has provided significant insight at a resolution unavailable to current experimental techniques, the uncertainty in model parameters, and the lack of experimental elucidation of these parameters across development, makes their broad application difficult.

Higher-level experimental techniques have attempted to address the nano vs. microdomain debate and have used the term “cooperativity” to make inferences as to the release modality. However, “cooperativity” has come to represent and describe a number of potentially unrelated characteristics of synaptic transmission. Mathematically, cooperativity is the degree of non-linearity present in an input-output system. In the case of neurons, the input-output equation is the relationship between Ca$^{2+}$ influx (I$_{Ca}$) and quantal release (Q), approximated by the following equation: $Q = a[Ca^{2+}]^m$, where the parameter
$m$ is defined as Ca$^{2+}$ cooperativity. The manner in which $m$ is generated (i.e. the experimental paradigm) may yield very different functional insight into the processes of synaptic transmission.

Traditionally, $I_{Ca}$ was varied by altering $[\text{Ca}^{2+}]_o$, producing varied output ($Q$) (Dodge & Rahamimoff, 1967). In these experiments the AP remains constant while the driving force for Ca$^{2+}$ ions is varied. Thus, on average, the same number of VGCCs open upon each stimulation but with variable $i_{Ca}$ as $[\text{Ca}^{2+}]_o$ is altered. With overwhelming consistency, $m$ has fallen into the range of 3-5 using this approach in a number of systems (Dodge & Rahamimoff, 1967; Smith et al., 1985; Augustine & Charlton, 1986; Stanley, 1986; Heidelberger et al., 1994; Landó & Zucker, 1994; Borst & Sakmann, 1996; Reid et al., 1998; Bollmann et al., 2000; Qian & Nobels, 2001). The interpretation of $m$ under these conditions is that it represents the number of Ca$^{2+}$ ions that cooperatively bind to the Ca$^{2+}$ sensor triggering a SV fusion event. These values appear to be in line with the number of Ca$^{2+}$ binding domains on synaptotagmin (Fernandez et al., 2001). In addition, graded photolysis of caged-Ca$^{2+}$ compounds, resulting in uniform increases in intraterminal $[\text{Ca}^{2+}]$, has reproduced the above $m$-values generated by electrophysiological means (Bollmann et al., 2000; Schneggenburger & Neher, 2000). The above experimental paradigms, which bypass or control for changes in the number of VGCCs contributing to the Ca$^{2+}$ signal, evaluate the “molecular cooperativity” or the cooperative binding of Ca$^{2+}$ ions to the Ca$^{2+}$ sensor (Gentile & Stanley, 2005).

In an alternative electrophysiological paradigm, the number of VGCCs contributing to SV release can be modulated while the driving force for Ca$^{2+}$ is held constant. In this sense, the non-linearity in output caused by recruiting successively greater numbers of VGCCs is represented by $m$ and has been termed the “Ca$^{2+}$ channel/domain cooperativity”. This version of $m$ is believed to reflect the number of VGCCs required to trigger SV release on average (Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005). Under these experimental conditions, molecular cooperativity should represent a binary condition that is satisfied when a sufficient number of VGCCs cooperate in creating a sufficiently large $I_{Ca}$. This condition also creates a theoretical upper limit, on the number of VGCCs that may contribute a Ca$^{2+}$ ion to the sensor, of five, assuming a five binding site model of synaptotagmin (Gentile & Stanley, 2005; Shahrezaei et al., 2006). That is, synaptotagmin may only accept one Ca$^{2+}$ ion from up to five distinct VGCCs. One caveat to this upper limit is that it assumes only one molecule of synaptotagmin is required for release when most predictions assume a significantly greater number (i.e. 3-15) (Weber et al., 1998; Montecucco et al., 2005; Jahn & Scheller, 2006). Indeed, $m$ values greater than 5 have been measured at the Calyx of Held (Fedchyshyn & Wang, 2005).

Interestingly, the variability observed in the Ca$^{2+}$ domain cooperativity across experimental conditions and preparations is much larger than that observed for molecular cooperativity. For example, $m$ may be less than 1 in the chick, 1-2 in the squid and frog NMJ, 3-4 in the mature calyx of Held, and 5-6 in the immature calyx of Held (Llinas et al., 1981; Charlton et al., 1982; Augustine & Eckert, 1984, Borst & Sakmann, 1999; Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005; Shahrezaei et al., 2006). In addition, the $[\text{Ca}^{2+}]_o$ appears to alter $m$ which may explain the observed differences between experimental systems (Gentile & Stanley, 2005). From these results, it is obvious that a number of different release modalities occur in nature and they appear to serve the primary function of releasing
SVs reliably (discussed further in Chapter 6 & 7). Some of the confusion surrounding the difference between “Ca$^{2+}$ domain” and “molecular” cooperativity arises from the numerical overlap, under certain conditions, of these distinct measures of $m$. For example at the calyx of Held, a Ca$^{2+}$ domain cooperativity value of 3-4 is measured from immature synapses in 2mM [Ca$^{2+}$], which aligns with the measured molecular cooperativity using the respective experimental paradigms (Borst & Sakmann 1996, Fedchyshyn & Wang, 2005). Given this overlap of $m$-values, the interpretation of $m$ must include consideration of its experimental derivation.

At the calyx of Held, molecular cooperativity appears to follow that of other systems suggesting that the Ca$^{2+}$ sensor is analogous. Also, as in other synapses (Luebke et al., 1993; Takahashi & Momiyama, 1993; Wheeler et al., 1994; Wu & Saggau, 1994; Mintz et al., 1995), the release of single vesicles at the young calyx of Held synapse depends on the cooperative action of overlapping Ca$^{2+}$ domains from many channels (Borst & Sakmann, 1996, Borst & Sakmann, 1999; Meinrenken et al., 2002). However, this requirement decreases with age, as $m$-values decrease by 50% by P16/17, suggesting that the nature of VGCC-SV coupling is a dynamic one (Fedchyshyn & Wang, 2005).

These changes in VGCC SV coupling with development, and the different arrangements of VGCCs and SVs observed throughout nature, suggest that certain arrangements may be an important component in conferring a synapses ability to function appropriately in its particular context.

1.5.3 Sources of Heterogeneity in Release Probability.

Understanding the dynamics of [Ca$^{2+}$] domains around VGCCs provides limits within which to consider other characteristics of the SV secretion apparatus. As discussed above, the arrangement of VGCCs necessary to release SVs is one of those characteristics. In addition to understanding upstream factors in SV release (i.e. Ca$^{2+}$ dynamics), use of downstream factors (i.e. $I_{\text{EPSC}}$) can also be instructive. At the calyx of Held, at least two different populations of SVs have been identified based on their kinetics and $P_r$ (Sakaba & Neher, 2001). One population is released rapidly with higher $P_r$, but is recycled more slowly, while the other population is released more slowly, but recycles quickly (Sakaba & Neher, 2001b). Similar “reluctant” and “willing” SVs have also been identified in other synapses (Moulder & Mennerick, 2005). In addition, different SVs appear to be modulated selectively by certain mechanisms. For example, in the calyx of Held, increases in [Ca$^{2+}$], appear to selectively convert slowly releasing SVs to rapidly releasing ones through a calmodulin-dependent mechanism, suggesting that SVs may be compositionally distinct from each other (Sakaba & Neher, 2001; Sakaba & Neher, 2001b). This heterogeneity in $P_r$ amongst SVs has sparked significant debate regarding the molecular or mechanistic differences between SVs that allow for their experimental differentiation.

Heterogeneity of $P_r$ is particularly important when performing calculations estimating quantal parameters based on whole-cell responses. A number of analytical methodologies have been developed to account for heterogeneity in $P_r$, while calculating the number of SVs in the RRP, under various conditions (i.e. moderate $P_r$) and limits (Meyer et al., 2001; Scheuss & Neher, 2001; Sakaba et al., 2002; Scheuss et al., 2002; Neher & Sakaba, 2001). In addition, heterogeneous $P_r$ may complicate the
interpretation of certain models of STP that may assume a homogeneous population of SVs such as early models of STD (Schneggenburger et al., 1999).

Besides the presynaptic mechanisms described here to account for heterogeneous P_r, postsynaptic mechanisms such as AMPAR desensitization can establish a “receptive” heterogeneity potentially misrepresenting the occurrence of presynaptic P_r heterogeneity. While these postsynaptic concerns can be minimized experimentally, we will not exhaustively explore their influence here.

There are two primary sources of presynaptic heterogeneity in P_r; Positional and molecular (Sakaba & Neher, 2001b). One of the current molecular hypotheses is that different populations of SVs may have different intrinsic sensitivities to [Ca^{2+}]. In a recent study by Wölfel et al., (2007), it was found that homogeneous increases in [Ca^{2+}], through flash-photolysis of caged-Ca^{2+}, could cause release with two distinct time courses. However, to adequately explain the observed STP, a component of use or Ca^{2+}-dependent sensitivity reduction had to be included in the model. Moreover, many of the capacitive traces recorded in this study showed no evidence of biphasic release (~45%), suggesting that the two observed release components may result from heterogeneity of the uncaged Ca^{2+} signal assumed to be homogeneous.

One of the most attractive explanations for the differing intrinsic SV sensitivities is that each may utilize different synaptotagmin isoforms as a Ca^{2+} sensor (Goda & Stevens, 1994). Indeed, synaptotagmin 2 has been identified at the calyx of Held, but not synaptotagmin 1 (Pang et al., 2006). However, no “slower” form of synaptotagmin (i.e. IV) has been identified in these synapses thus far. Similarly, it is possible that the protein composition of individual SVs varies such that the release sites they occupy are differentially targeted for modulation. With the myriad of proteins found associated with SVs, this possibility appears reasonable (Burré & Volknandt, 2007).

A number of potential explanations exist describing use or Ca^{2+}-dependent alterations in P_r required of the Wölfel et al., (2007) model. Exhaustion of release machinery (Waldeck et al., 2000; also see Lin & Faber, 2002), lateral inhibition of neighbouring release sites (Stevens & Wang, 1995; Dobrunz et al., 1997), and adaptation of the release apparatus (Hsu et al., 1996) have all been shown to alter the P_r of SVs, presumably through changes to the Ca^{2+} sensitivity of release. Although these mechanisms may operate in other experimental systems (as above), none have been shown to operate at the calyx of Held. In fact, evidence exists to suggest that the Ca^{2+} sensitivity of the release machinery is highly stable during a number of forms of STP and stimulation paradigms (Felmy et al., 2003; Bollmann & Sakmann, 2005; Wadel et al., 2007). In addition, recent work suggests that only a small portion of SVs appear to have an intrinsic or molecular tendency to be slowly releasing suggesting that further investigation as to the underlying cause of heterogeneous P_r is required (Wadel et al., 2007).

While perhaps not clearly molecular, heterogeneity in the membrane potential across release sites may account for heterogeneity in P_r. In classic work from the squid giant synapse, Augustine et al. (1985a & 1985b) found that space clamp limitations could impact the properties of VGCCs at different points on the membrane. Likewise, passive spread of APs, as occurs at the calyx of Held (Leão et al., 2005), may result in regions of VGCCs where membrane potential variations establish lower open probability than at others. This would result in SVs with different P_r through altered Ca^{2+} dynamics.
The second main hypothesis set forth to explain heterogeneous PB is the positional model where the different VGCC-SV separation distances results in SVs with differing PB (Roberts, 1994; Klingauf & Neher, 1997; Naraghi & Neher, 1998; Rozov et al., 2001; Felmy et al., 2003; Trommershäuser et al., 2003; Bollmann & Sakmann, 2005; Wadel et al., 2007). The essential feature of this model is that the magnitude of [Ca^{2+}] domains varies significantly with distance from the VGCC or VGCC cluster. In support of this argument are modelling studies which show that even small changes to the separation distance between VGCCs and SV, from 30-300nm, can produce a range of PB from <0.01 to 1 (Meinrenken et al., 2002). In addition, it was found recently at the calyx of Held that most (~80%) of SVs have fast intrinsic release kinetics, although the proportion of fast releasing SVs varies depending on the Ca^{2+} signal (Wadel et al., 2007). This suggests that the different kinetics observed in release arises primarily from the magnitude of Ca^{2+} signal they experience (Wadel et al., 2007). This is further strengthened by the finding that the rate of SV fusion is dependent on the rate of Ca^{2+} ion binding to the Ca^{2+} sensor rather than the fusion rate of the machinery itself (Felmy et al., 2003).

Two primary arguments exist against the positional model of heterogeneity. The first relies on the assumption that if many (i.e. 60) VGCCs interact to trigger SV fusion, then the spatiotemporal nature of the [Ca^{2+}] domain should matter less than the sensitivity of the release machinery (Wu & Borst, 1999). However, the actual estimate of the number of VGCCs mediating SV release has significantly decreased since this interpretation (Meinrenken et al., 2002). Secondly, early studies could not use changes in minimal SD to determine whether fast or slowly releasing SVs had been released because the diffusion time of Ca^{2+} ions was thought to comprise a small component of the total delay (Adler et al., 1991; Yamada & Zucker, 1992; Borst & Sakmann, 1996). However, recent advancements in determination techniques of SD (Fedchyshyn & Wang, 2007) and further maturation of experimental techniques have shown that changes in SD are visible when the [Ca^{2+}] is altered (Bollmann et al., 2000; Bollmann & Sakmann, 2005; Fedchyshyn & Wang, 2007; Chapter 5 & 6).

Positional heterogeneity was hypothesized at a lower-level by Shahrezaei & Delaney (2004) when they examined the impact of the Ca^{2+} sensor position around a SV on PB. The diffusion barrier of the SV itself could significantly alter the PB with changes of as little as 5nm in Ca^{2+} sensor-channel distance, resulting in a 3-fold increase in PB.

At a higher-level, heterogeneity in PB has been shown between synapses of the same neuron. This type of heterogeneity has been ascribed to the size of the AZ, the number of release sites, AZ morphology, VGCC density, and the size of the RRP (Dobrunz & Stevens, 1997; Murthy et al., 1997; Rosenmund et al., 1993; Rozov et al., 2001; Brenowitz & Regehr, 2007). There is even some evidence suggesting that neighbouring synapses influence each other’s PB (Murthy et al., 1997).

Based on these data, it is difficult to deny the role relative positioning of the VGCC and SV play in determining PB, and its heterogeneity. While there may be a component of PB determined intrinsically by the distinct make-up or modulation of certain SVs, the tight control of [Ca^{2+}] domains, and the evidence supporting positional heterogeneity, provides a compelling explanation for this currently unresolved property of SV release.
1.5.4 Kinetics of Synaptic Vesicle Release & Sources of Delay.

In addition to the relationship between Ca\textsuperscript{2+} influx and SV release, the delays observed in the SV release process can be instructive in elucidating the nature of the steps underlying it. Below we will consider only those neuronal delays between the stimulated generation of an AP and the point of maximal postsynaptic response (I_{EPSC}), with a particular focus on the presynaptic processes that contribute to establishing these delays.

The general steps in the propagation of an AP from pre- to postsynaptic neuron are; generation of an AP at a node of Ranvier through external stimulation, conduction of the action potential to a presynaptic terminal, the opening of VGCCs in response to depolarization evoked by the AP, the entry and transiting of Ca\textsuperscript{2+} ions to the Ca\textsuperscript{2+} sensor, the binding of those ions to the sensor, the action of the sensor in promoting fusion of the vesicular and target membranes, the diffusion of a neurotransmitter out of the SV and across the synaptic cleft, the binding of transmitter to postsynaptic ionotropic receptors, and their kinetics in generating a postsynaptic current (Südhof, 2004). Each of these components carries an intrinsic delay that may be static or dynamic depending on the conditions and on previous activity (Lin & Faber, 2002). Electrophysiologically, these components have been very difficult to separate, particularly in the mammalian CNS, where inaccessible presynaptic terminals limit recording of presynaptic events in time. At the calyx of Held, simultaneous pre- and postsynaptic whole-cell recordings, in either voltage- or current-clamp, allow for the resolution of a number of delay parameters. Here we have separated them into conduction delay, synaptic delay, and response-rise delay (Fedchyshyn & Wang, 2007).

Conduction delay (CD) encompasses the processes of AP generation, and AP propagation from the axon to the terminal. APs propagate passively from the site of their generation to the nerve terminal at a rate which depends on the cable properties of the axon (Waxman, 1980). AP conduction velocity is directly proportional to the thickness of the myelin insulation, the temperature, and axon diameter. In addition the internodal distance, the Na\textsuperscript{+} channel nodal density, the composition/resistivity of the axonal contents, and the ratio of myelin thickness/axon diameter/internodal distance also determine the conduction velocity (Waxman, 1980). At the calyx of Held, conduction delay is very short in mature synapses (~0.1ms) owing to wide diameter axons and the developmental increase in myelin covering (von Gersdorff & Borst, 2002; Fedchyshyn & Wang, 2007).

Synaptic delay (SD) is classically defined as the time delay between the arrival of the presynaptic AP and the onset of a postsynaptic response (Katz & Miledi, 1965b). Included in this delay are all processes from the opening of VGCCs to the activation of postsynaptic ionotropic receptors, making it the most interesting in the study of SV release. As a result of the number of processes compiled into this delay, it is often difficult to determine which components are limiting, which may be plastic, and which are potential targets of modulation (Lin & Faber, 2002). Another complication in using SD as a means of investigating SV release is that its already brief duration, ~200\mu s in the squid and calyx of Held at their respective body temperatures, make detecting changes largely a matter of analytical accuracy (Llinás et al., 1976; Fedchyshyn & Wang, 2007). Indeed, this lack of accuracy may underlie the common assumption that SD is invariant (discussed below). Recent studies at the calyx of
Held, show that the binding kinetics of Ca\(^{2+}\) to the Ca\(^{2+}\) sensor is the limiting factor in determining the delay of SV release (Felmy et al., 2003). This allows exclusion of this step in determining how modifications to Ca\(^{2+}\) influx, for example, alter SD. In addition, since the amount of neurotransmitter is approximately quantized (also see Wu et al., 2007), the rate of diffusion across the synaptic cleft should be approximately equal across all release sites assuming primarily monoquantal release and a continuous cleft separation (Trommershäuser et al., 1999; Savtchenko & Rusakov, 2007). Diffusion of neurotransmitter across CNS synaptic clefts is thought to be very rapid, on the order of µs, making its contribution to overall SD minimal. Thus any variability in SD should be primarily determined by the nature of the AP in determining VGCC opening (Benoit & Mambrini, 1970), the rate at which Ca\(^{2+}\) ions bind to the Ca\(^{2+}\) sensor, and the kinetics of postsynaptic receptor activation. In the case of AMPARs, the activation rate depends on the rate of rise of glutamate concentration and the intrinsic kinetics of the AMPAR itself (Jonas & Sprutson, 1994). The spatial distribution of AMPARs and their interaction with varying glutamate concentration profiles is believed to underlie the slower rise-time of compound I\(_{\text{EPSC}}\), with the average AMPAR-mediated quanta having a rise-time of ~0.5ms or less (Trommershäuser et al., 1999). Thus, the delay to onset of the compound I\(_{\text{EPSC}}\) reflects the detection of the first released quanta (i.e. from those AMPARs in direct apposition of presynaptic release sites) including their <0.5ms activation time, but excludes the variability of AMPAR activation caused by variability in AMPAR localization (Trommershäuser et al., 1999).

Response-rise delay (RRD) is measured from the onset of the postsynaptic I\(_{\text{EPSC}}\) to its peak (Fedchyshyn & Wang, 2007). This delay takes into account the variability of glutamate binding to AMPARs of differing appositions to release sites. It can also be indicative of the synchrony of release events or the “sharpness” of the glutamate transient experienced at the AMPARs.

In many classical studies of synaptic transmission, it was thought that the kinetics of synaptic transmission were extremely stable and sensitive to few experimental manipulations (Katz & Miledi, 1965; Barrett & Stevens, 1972; Datyner & Gage, 1980; Parnas et al., 1989). More recently, this assumption of invariance has come under scrutiny with a number of accounts finding that SD was in fact dynamic and depended on kinetic changes in many of the processes outlined above (Waldeck et al., 2000; Vyshedskiy et al., 2000; Fedchyshyn & Wang, 2007). However, targeting the specific processes and under what conditions SD changes is only somewhat understood (Lin & Faber, 2002).

Some components of delay appear to be relatively resistant to changes in experimental paradigm. At the calyx of Held, CD appears to be consistent independent of [Ca\(^{2+}\)]\(_{o}\) but appears to lengthen slightly during high-frequency repetitive activity (Fedchyshyn & Wang, 2007). This may arise due to axonal fatigue caused by the transient build-up of [K\(^+\)], or decreases in [Na\(^{+}\)]\(_{o}\) at the nodes of Ranvier or inactivation of either of these channel types. However, this temporal plasticity appears to depend only on activity and temperature rather than the stimulation frequency or [Ca\(^{2+}\)]\(_{o}\) (Fedchyshyn & Wang, 2007).

In contrast, SD and RRD both appear to depend on presynaptic Ca\(^{2+}\) both during sustained activity and in response to single APs (Fedchyshyn & Wang, 2007; Chapter 5 & 6). Indications of this dependence have been evident since the studies of Benoit & Mambrini (1970), which found
pharmacologically that AP duration (half-width) was inversely proportional to SD. This suggested that the impact of changes in membrane potential time-course impacted the \( I_{Ca} \) such that SD and the time-course of release were altered. Since this work, separate sets of studies have provided evidence showing that SD can be both shortened and lengthened in certain systems and under appropriate conditions. These conditions appear to favour modulation of Ca\(^{2+}\) dynamics, and thus \( P_r \), as the means of eliciting changes in SD. When Ca\(^{2+}\) is rapidly elevated, either through the use of long voltage steps or through flash photolysis of cages Ca\(^{2+}\) compounds, SD can be measurably decreased (Vyshedskiy \& Lin, 1997; Borst \& Sakmann, 1999; Vyshedskiy et al., 2000; Bollmann \& Sakmann, 2005). Conversely, decreases in \( P_r \), brought about through depletion of SVs, or following tetanic stimulation, causes increases in SD (Wu \& Borst, 1999; Habets \& Borst, 2005; Fedchyshyn \& Wang, 2007; Kim et al., 2007). As described above, the \( P_r \) of SVs is heterogeneous depending primarily on differences in their interactions with Ca\(^{2+}\) ions which may underlie the bidirectional modulation of SD (Bollmann et al., 2000; Meinrenken et al., 2002; Meinrenken et al., 2003; Trommersh"{a}user et al., 2003; Fedchyshyn \& Wang, 2005).

In experiments showing a shortening in SD, manipulation of [Ca\(^{2+}\)] domains appears to be a common requirement. Due to the dependence of SV release kinetics on Ca\(^{2+}\) binding to the Ca\(^{2+}\) sensor, increasing the [Ca\(^{2+}\)] domain at the Ca\(^{2+}\) sensor effectively shortens SD (Felmy et al., 2003; Chapter 5 \& 6). In addition, the high sensitivity of the Ca\(^{2+}\) sensor implies that small changes to [Ca\(^{2+}\)] domains and their duration will have significant impact on the binding kinetics of the sensor (Yamada \& Zucker, 1992; Bollmann et al., 2000; Schneggenburger \& Neher, 2000; Shahrezaei \& Delaney, 2005). Different sized [Ca\(^{2+}\)] domains will also be impacted to differing extents by endogenous buffers within the AZ, thereby affecting SD (Naraghi \& Neher, 1997; Shahrezaei \& Delaney, 2004; Meinrenken et al., 2002; Meinrenken et al., 2003). Since most other processes included in SD (i.e. neurotransmitter diffusion) should be relatively constant independent of the inter-terminal Ca\(^{2+}\) dynamics, changes in SD provide an alternative means of monitoring the processes of excitation-secretion coupling.

Interestingly, while the dynamics of Ca\(^{2+}\) domains appear to determine the timing of SV release to some extent, Ca\(^{2+}\) itself also appears to underlie the prolongation in SD; however, the exact mechanism underlying this phenomenon is not yet clear. During repetitive activity, or under other conditions of SV depletion, SD increases (Wu \& Borst, 1999; Fedchyshyn \& Wang, 2007). One theory is that prolongations in delay result from the same mechanism that reduces \( P_r \) during STD, being the unavailability of SVs. As SVs are recruited from locations further and further from the VGCCs and AZs, the diffusion time for Ca\(^{2+}\) increases and the “sharpness” of the [Ca\(^{2+}\)] domain they experience is reduced, delaying the binding of Ca\(^{2+}\) to the Ca\(^{2+}\) sensor (Meinrenken et al., 2002; Habets \& Borst, 2005; Fedchyshyn \& Wang, 2007). Reducing build-up of Ca\(^{2+}\) within the terminal decreases the rate at which SD is prolonged and the rate of SV depletion suggesting a link between these two processes (Fedchyshyn \& Wang, 2007). As a result of the increasing heterogeneity in the [Ca\(^{2+}\)] domains experienced by SVs of increasing distance from the VGCCs, increasing asynchrony of release (jitter of \( I_{EPSC} \) timing) is also expected and observed (Fedchyshyn \& Wang, 2007). The competing hypotheses suggest that increased delay is the result of changes in Ca\(^{2+}\) sensitivity of the release machinery based
on activity and/or Ca\textsuperscript{2+} from previous events (Stevens & Wang, 1995; Hsu et al., 1996; Wu & Borst, 1999; Wölfel et al., 2007). However, these possibilities seem unlikely given recent findings from the calyx of Held showing that the sensitivity of the immature release apparatus is maintained even when the synapse is depressed and our finding that increases in [Ca\textsuperscript{2+}]\textsubscript{i} alone cannot increase SD without SV depletion (Wadel et al., 2007; see Chapter 6).

Also of note is the observation that SD decreases with development at the calyx of Held (Taschenberger & von Gersdorff, 2000; Taschenberger et al., 2005). However, it remains unknown which processes in SV release underlie the decrease. The determination of the underlying processes in developmental SD shortening is complicated by the concurrent developmental decreases in AP width, which alters Ca\textsuperscript{2+} dynamics and affects SD (Taschenberger et al., 2002; Fedchyshyn & Wang, 2005). Circumventing this issue by using the same input AP waveform to trigger similar I\textsubscript{Ca} and I\textsubscript{EPSC} shows that the difference in SD remains independent of AP waveform, which may have significant influence on the kinetics of VGCCs (Pattillo et al., 1999; Chapter 5). These data suggest that developmental differences in SD arise from differences in [Ca\textsuperscript{2+}] dynamics, possibly due to distinct VGCC-SV coupling across development (Fedchyshyn & Wang, 2005).

While the exact mechanisms remain unconfirmed, it is clear that Ca\textsuperscript{2+}, whether by altering the molecular properties of the release machinery or by variances in its dynamics at the AZ, underlies changes in SD both within a synapse and across development. With new techniques available for the high-accuracy determination of delays at synapses like the calyx of Held (Fedchyshyn & Wang, 2007), the study of SD provides an alternate means of indirectly observing the SV release process.

### 1.6 SHORT-TERM PLASTICITY

#### 1.6.1 Short-Term Depression.

Short-term depression (STD) may be defined as the activity-dependent reduction synaptic response occurring over relatively short periods of time (i.e. ms) (Zucker & Regehr, 2002). A number of mechanisms have been proposed and demonstrated, in various preparations, to account for STD (von Gersdorff & Borst, 2002; Zucker & Regehr, 2002). Here we will focus on pre- and postsynaptic mechanisms occurring within a single synapse and exclude explicit discussion of potential ultrastructural mechanisms underlying expression of short-term plasticity (STP) such as changes to the number of AZs, or the number of docked SVs (see Xu-Friedman & Regehr, 2004).

At the calyx of Held-MNTB synapse, STD of I\textsubscript{EPSC} is the dominant form of STP at most frequencies and under typical recoding conditions (i.e. 2mM [Ca\textsuperscript{2+}]\textsubscript{i}) (Borst et al., 1995; von Gersdorff et al., 1997; Wang & Kaczmarek, 1998). Depending on the frequency of activity, and on the developmental stage of the synapse (see below), different mechanisms contribute to STD to differing degrees. At high-frequencies (>100Hz), STD is believed to arise primarily due to depletion of SVs from the RRP and desensitization/saturation of postsynaptic AMPARs (Neher & Sakaba, 2001; Joshi & Wang, 2002; Scheuss et al., 2002; Taschenberger et al., 2002, Taschenberger et al., 2005; Wong et al., 2003). In the
latter case, underestimation of the actual number of quanta released occurs as the $I_{\text{EPSC}}$ is no longer a faithful read-out of presynaptic release (Scheuss et al., 2002; Wong et al., 2003; Taschenberger et al., 2005). Addition of CTZ or kynurenic acid can minimize the extent to which these postsynaptic mechanisms occur (Neher & Sakaba, 2001; Sakaba et al., 2002). Under these conditions, the decrease in $P_r$ observed during STD appears to be exclusively presynaptic, the mechanisms underlying which are under some debate. Decreased $P_r$ can arise from the activity-dependent recruitment of SVs from sites more and more distant from the release sites (Fedchyshyn & Wang, 2007; Wadel et al., 2007), or it may arise from the selective depletion of highly Ca$^{2+}$-sensitive SVs followed by less Ca$^{2+}$-sensitive ones (Wölfel et al., 2007). In either case, the depletion of SVs underlies the reduction in $I_{\text{EPSC}}$ with activity in the absence of desensitization and saturation. Maintaining a simple model of SV depletion is complicated by the fact that replenishment of SVs is also activity-dependent, and can occur on very rapid time scales, affecting the rate of STD and the extent to which it occurs (Wang & Kaczmarek, 1998; Sakaba & Neher, 2001; Sakaba, 2006). In addition, the heterogeneous $P_r$ of SVs causes the rate and extent of STD not to follow the anticipated 1/f rule expected of a simple depletion model. Upstream of SV release, APs at the calyx of Held show slight broadening and attenuation during high-frequency firing which should result in attenuation of $I_{\text{Ca}}$. However, $I_{\text{Ca}}$ are generally maintained despite the change in AP shape, most likely due to concurrent activity-dependent facilitation of $I_{\text{Ca}}$ (Tsujimoto et al., 2002; Inchauspe et al., 2004; Ishikawa et al., 2005; Inchauspe et al., 2007). These findings suggest that the source of STD at the calyx of Held resides downstream of changes in presynaptic AP waveform and the resultant $I_{\text{Ca}}$ (Borst & Sakmann, 1999).

At lower frequencies (<30Hz), Ca$^{2+}$/activity-dependent inactivation of VGCCs is a significant source of STD while SV depletion is believed to contribute little (Forsythe et al., 1998; Xu & Wu, 2005).

A number of GPCRs may also contribute to the reduction of $I_{\text{Ca}}$ (i.e. mGluRs) and STD, however, the extent of their contribution appears minimal (von Gersdorff et al., 1997; see Section 1.3). Adaptation or exhaustion of the release machinery has also been hypothesized and demonstrated in other synapses as underlying STD (Stevens & Wang, 1995; Hsu et al., 1996; Dobruntz et al., 1997; Dittman & Regehr, 1998; Dittman et al., 2000), however, these mechanisms have been largely excluded at the calyx of Held (Wu & Borst, 1999; see Chapter 6).

Given the above, it appears that the finite size of the RRP is the limiting factor in the maintenance of continual high-frequency synaptic transmission at the calyx of Held. While postsynaptic mechanisms exacerbate this effect further, the narrowed APs, rapid diffusion, and rapid SV recycling observed at physiological temperatures, would decrease the contribution of these mechanisms (Yang & Wang, 2006; Kushmerick et al., 2006). Furthermore, the delayed kinetics of $I_{\text{EPSC}}$ late in stimulus trains and the dependence of SD progression rate on stimulation frequency suggests that the decreased $P_r$ observed during STD arises most probably from the successive recruitment of distant SVs following depletion of those located close to the AZ (Fedchyshyn & Wang, 2007; Wadel et al., 2007).
1.6.2 Short-Term Facilitation.

The transient increase in SV \( P \) with successive stimulation is termed short-term facilitation (STF) and is the prominent form of STP at synapses like the parallel fibre to Purkinje cell synapse (Dittman et al., 2000). At the calyx of Held-MNTB synapse, STF is generally observed only under conditions of experimentally reduced \( P \), or when postsynaptic mechanisms of STD are eliminated (Barnes-Davies & Forsythe, 1995; Borst et al., 1995; Sakaba & Neher, 2001a; von Gersdorff & Borst, 2002; Felmy et al., 2003; Wong et al., 2003). Mechanistically, part of STF can be attributed to facilitation of \( I_{Ca} \) and part to steps downstream of \( Ca^{2+} \) influx. Activity-dependent \( I_{Ca} \) facilitation appears to occur only to P/Q-type VGCCs and appears to require \( Ca^{2+} \) (Cuttle et al., 1998; Tsujimoto et al., 2002; Inchauspe et al., 2004; Ishikawa et al., 2005). To date, the only mechanism proposed to account for \( I_{Ca} \) facilitation involved the detection of increased \( [Ca^{2+}]_i \) by NCS-1 (Tsujimoto et al., 2002). Generally, \( I_{Ca} \) late in high-frequency trains increases at most 20%, compared to the first \( I_{Ca} \) in the series, when facilitated. This increase can have a significant impact on \( P \), due to the highly non-linear relationship between \( I_{Ca} \) and SV release (Dodge & Rahamimoff, 1967; Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005; Wu & Xu, 2007). Despite this non-linearity, \( I_{Ca} \) facilitation cannot account for all of the observed \( I_{EPSC} \) facilitation observed at the calyx of Held (Felmy et al., 2003; Wong et al., 2003; Xu & Wu, 2007). Postsynaptic mechanisms describing STF have also been proposed at other synapses, such as activity-dependent unblock of AMPARs by spermine, but have not been proven to act at the calyx of Held-MNTB synapse (Rozov & Burnashev, 1999).

The most popular hypothesis for explaining STF is the “residual \( Ca^{2+} \) hypothesis” which, since its establishment (Katz & Miledi, 1965), has provoked much debate. The controversy revolves less around whether \( Ca^{2+} \) ions are involved in transiently increasing \( P \), but more around the particular means by which they do so (Zucker, 1999; Zucker & Regehr, 2003). The original formulation of the hypothesis assumed that if an initial AP was followed in rapid succession by another AP, the \( [Ca^{2+}]_i \) remaining from the first AP and the \( Ca^{2+} \) influx from the second AP would summate linearly to create a larger \( Ca^{2+} \) signal thereby facilitating release (Katz & Miledi, 1965). Some of the most compelling evidence in support of this idea comes from the observation that the addition of exogenous buffers like EGTA eliminate STF in a number of preparations, presumably due to chelation of residual \( Ca^{2+} \) (Zucker & Regehr, 2002). With better understanding of the stoichiometry of the \( Ca^{2+} \) sensor, “bound models” of facilitation were and continue to be proposed. In these models, partial occupancy of the \( Ca^{2+} \) sensor is maintained following an initial AP, facilitating release for subsequent events (Stanley, 1986; Yamada & Zucker, 1992; Bertram, 1997; Matveev et al., 2006). These models were devised largely to account for the difference in time course between the decay of STF and the decay of residual \( Ca^{2+} \) in some systems (Parnas & Parnas, 1994). However, the apparent sensitivity of the \( Ca^{2+} \) sensor (~100 \( \mu \)m), which could be reached in close proximity to the VGCC pore, made the low residual \( [Ca^{2+}]_i \) (a few \( \mu \)m) appear an insignificant addition to the domain \( [Ca^{2+}]_i \) (Simon & Llinás, 1985; Neher, 1998). The paradox surrounded how the sensor could remain unsaturated, in the presence of 100\( \mu \)M \( Ca^{2+} \), and detect additions of residual \( [Ca^{2+}]_i \) of a few \( \mu \)m at the same time? A variation on this hypothesis was proposed in which a distinct “facilitation site”,
physically separate and of high-affinity than the release site, was capable of detecting small changes in Ca$^{2+}$ (Yamada & Zucker, 1992).

Clarification was gained in more recent experiments performed at the calyx of Held-MNTB synapse in which the Ca$^{2+}$ sensor was found to have an affinity closer to 10-25μM (Bollmann et al., 2000; Schneggenburger & Neher, 2000). These findings implied that the Ca$^{2+}$ sensor was capable of monitoring changes in [Ca$^{2+}$], that were much finer than originally thought. Further evidence from the frog NMJ suggested that, despite the higher sensitivity, the brief time course of I$_{\text{Ca}}$, and the presence of Ca$^{2+}$ buffering, prevents saturation of the Ca$^{2+}$ sensor (Shahrezaei & Delaney, 2005). Due to the accessibility of the calyx of Held nerve terminal, it was established that elevations in residual Ca$^{2+}$ persist for tens of ms following an AP, on the order of the length of time STF is observed in these synapses (Helmchen et al., 1997; Felmy et al., 2003). This suggested that there may be no requirement for an additional Ca$^{2+}$ sensing site in modeling residual Ca$^{2+}$. However, in accounting for STF using residual Ca$^{2+}$, there appears to be a small component of I$_{\text{EPSC}}$ potentiation (0.4-fold) which cannot be accounted for by residual Ca$^{2+}$ alone (Felmy et al., 2003). While other mechanisms, such as a difference in intrinsic Ca$^{2+}$ sensitivity of some SVs (Wölfel et al., 2007) may account for the discrepancy, the most likely cause is the local saturation of Ca$^{2+}$ buffers (Klingauf & Neher, 1997; Naraghi & Neher, 1997; Sakaba & Neher, 1998; Neher, 1998, see also Burnashev & Rozov, 2005). When buffers become saturated, the buffered Ca$^{2+}$ signal at the Ca$^{2+}$ sensor is potentiated resulting in higher P$_{r}$ for a population of SVs that may not otherwise experience suprathreshold Ca$^{2+}$ signals (Neher, 1998; see section 1.5).

While further elucidation of the underlying mechanisms of STF is required, it is clear that Ca$^{2+}$ dynamics is a key factor in its expression. During high-frequency activity, interplay between the mechanisms of STF and STD determine the overall P$_{r}$ of the synapse and ultimately the response generated in the postsynaptic neuron (Dittman et al., 2000). It is clear that the proportion of STD and STF will depend on the experimental system, conditions, and the intensity of activity, making generalized modelling of these intertwined processes challenging.

1.6.3 Short-Term Plasticity and Development.

The development of the calyx of Held-MNTB synapse has been well characterized over the critical period bracketing sensory onset (P8-P18) (Chuma & Ohmori, 1998; Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; Joshi et al., 2004; Fedchyshyn & Wang, 2005; Koike-Tani, et al., 2005; Leão et al., 2005; Yang & Wang, 2006; Youssoufian et al., 2005; Taschenberger et al., 2005). As a result, the underlying mechanisms of STP have also been relatively well characterized at this synapse (von Gersdorff & Borst, 2002; Schneggenberger & Forsythe, 2006). However, most of this understanding has come from immature synapses which exhibits distinct patterns of STP from mature synapses (Taschenberger & von Gersdorff, 2000; Taschenberger et al., 2002; Taschenberger et al., 2005). STD is generally found to be more prominent in immature synapses, which stems from their higher measured P$_{r}$ (Taschenberger & von Gersdorff, 2000; Xu et al., 2007). However, this is largely due to the wider APs, and resultant larger I$_{\text{Ca}}$, that is typical of synapses at this developmental stage (Taschenberger & von Gersdorff, 2000; Fedchyshyn & Wang, 2005). A number of mechanisms
downstream of APs also differentially influence the patterns of STP observed at this synapse with development.

The expression of endogenous Ca\(^{2+}\) buffers (i.e. parvalbumin) has been shown to increase markedly directly preceding the onset of hearing (Lohman & Friauf, 1996; Müller et al., 2007), however, following this no evidence exists as to whether this trend continues. As discussed above, Ca\(^{2+}\) buffers shape the dynamics of [Ca\(^{2+}\)] domains implying that their concentrations define to what extent, for what duration, and with what reach Ca\(^{2+}\) elevations will occur within the terminal. During repetitive activity, it is likely that rapid repeated influxes of Ca\(^{2+}\) to the same regions can overwhelm the endogenous buffering capacity leading to increased [Ca\(^{2+}\)], and possibly STF (Naraghi & Neher, 1997; Felmy et al., 2003). If the developmental expression of parvalbumin were to continue to increase, and given its similar properties to EGTA, it is likely that STF would be attenuated with development, assuming VGCC-SV distances on the order of 100nm (Naraghi & Neher, 1997; Meinrenken et al., 2002). Interestingly, most studies employing whole-cell recording arrangements at the calyx of Held would cause rapid dialysis of endogenous buffers making developmental changes in this synaptic property unobservable (Felmy & Schneggenburger, 2004; Müller et al., 2007).

Related to the above are developmental changes in VGCC-SV coupling which have been shown to tighten with development (Fedchyshyn & Wang, 2005). The type of release modality impacts a number of synaptic parameters which influence the observed patterns of STP. For example, VGCC-SV separation distance is a primary factor in determining the effectiveness of endogenous buffering of Ca\(^{2+}\) (Naraghi & Neher, 1997). Loosely-coupled SVs are likely to experience buffered Ca\(^{2+}\) diffusion, which attenuates the Ca\(^{2+}\) signal, making it more likely that subthreshold Ca\(^{2+}\) signals will be experienced. In contrast, tight coupling may place the Ca\(^{2+}\) sensor in an unbuffered region of high Ca\(^{2+}\). Since tight coupling increases P\(_{in}\), given identical Ca\(^{2+}\) signals, mature synapses should show greater STD than immature ones. Furthermore, the loose coupling of SVs and the heterogeneity of P\(_{in}\), especially in immature synapses, impart the necessary structural arrangement for greater STF to occur. The role of VGCC-SV coupling in STP is discussed at length in Chapter 6.

Of postsynaptic factors, desensitization and saturation of AMPARs are the primary determinants of STP. At the immature calyx of Held, it is estimated that release of only 30% of the RRP is required to cause saturation of AMPARs (Sun & Wu, 2001). However, although not explicitly investigated at this synapse, the degree to which AMPA saturation occurs is believed to decrease with development due to the more rapid clearance of glutamate. Desensitization of AMPARs is likely increasing the apparent STD in immature synapses and having a lesser effect on mature ones. As the calyx of Held achieves its fenestrated mature morphology, better diffusion into and out of the synaptic cleft is achieved resulting in the improved clearance of glutamate and replenishment of Ca\(^{2+}\) (Taschenberger & von Gersdorff, 2000). This implies that as development progresses, the mechanisms underlying STP shift to employ those of presynaptic origin over those of the postsynapse. This is advantageous as it shifts modulation to the input side of the synapse where non-linearities can amplify small changes in I\(_{Ca}\). In addition, attenuation of postsynaptic sources of STP makes the MNTB neuron a more faithful reproducer of presynaptic output. Therefore, released SVs are not “lost” due to unfaithful or “deaf” postsynaptic reception. The
above may serve as an adaptation which allows for the faithful reproduction of higher frequency signals and their effective modulation in the mature synapse.

1.7 PERSPECTIVE & OVERVIEW

1.7.1 In the Context of Development.

The appeal of the calyx of Held-MNTB synapse as a model system stems primarily from the accessibility of the presynaptic terminal to electrophysiological experimentation (see section 1.2). However, the characteristic of this synapse that has been less-explored in the literature is its presynaptic development. The calyx of Held nerve terminal is in fact accessible to electrophysiological recording during a period (from ~P5 to P20) which brackets the onset of hearing in the animal (~P11) and the associated onset of sensory-driven activity in the system. This allows for a remarkable opportunity to study single synaptic development and also provokes questioning the causal relationships underlying the many synaptic changes occurring over this period.

To date much of the focus in this system has been aimed at uncovering the fundamental properties of excitation-secretion coupling, buffered Ca$^{2+}$ diffusion, the nature of the RRP, and STP in a mammalian nerve terminal (Schneggenburger & Forsythe, 2006). However, most studies have been performed in synapses prior to the onset of sensory activity (<P10) and the many correlated synaptic changes that coalesce into a synapse of functional maturity. The preference to experiment at this developmental stage arises mainly due to technical difficulties experienced when studying more mature synapses. With development, myelination increases significantly, decreasing tissue clarity under the microscope and making visual identification of the calyx of Held difficult. In addition, the morphology of the calyx itself becomes less permissive to whole-cell electrophysiological recordings as it becomes more fenestrated following the onset of hearing.

However, with functional maturity in the system being reached at least one week after hearing onset, the conclusions drawn from experiments prior to this period must be cautiously extended beyond the framework of a decidedly undeveloped synapse.

The days following hearing onset are particularly dynamic in terms of morphological development and the modification of synaptic properties (see section 1.2.2). Much of the developmental work performed at the calyx of Held has used synapses in the midst of this period (P13-14) as "mature", again due to the technical difficulty of acquiring whole-cell recordings from fenestrated terminals. Synapses of this age still exhibit many immature characteristics, such as smaller mEPSC amplitudes (Yamashita et al., 2003), and may not provide a sufficient separation from the "immature" group to uncover adaptations occurring in subsequent days. Thus, the instability of synapses at this time in development and the narrow window of observation have led to underestimation in the difference between the synaptic properties of the calyx of Held synapse before the onset of hearing and into maturity. In addition, early studies compiled large age ranges which smeared rapid developmental changes making detection of subtle alterations impossible (Forsythe et al., 1998).
Due to the above, a large gap existed in our understanding of the mature presynaptic terminal at the time when the works that comprise this thesis were undertaken. Advanced modelling has led to a deep understanding regarding the SV secretion process in immature synapses (Meinrenken et al., 2002), but with few of the necessary parameters available from the mature synapse, the same level of prediction could not be gained. A decrease in presynaptic AP duration had been characterized with development while \( I_{\text{EPSC}} \) amplitudes had been shown to increase in concert. However, little evidence existed as to the mechanism underlying this paradoxical observance (Taschenberger & von Gersdorff, 2000).

At a higher level, we did not understand why these changes would be advantageous to the mature synapse, the functioning of the sound localization circuit, or their impact on the development of proper signal processing. While the characteristics of the MNTB neuron had been well studied into maturity, the developmental changes occurring in the MNTB needed to be considered in the context of a dynamic nerve terminal. For example, AMPAR desensitization is known to decrease with development owing partially to a change in GluR subunits but also to presynaptic properties such as calyceal fenestration providing greater diffusional access/egress (Otis et al., 1996; Joshi et al., 2004; Koike-Tani et al., 2005).

Some of the most intriguing high-level questions at the calyx of Held-MNTB synapse had surrounded the issue of causality. Only recently has the role of activity been investigated in the context of synaptic development, but again primarily in the postsynaptic MNTB neuron (Youssoufian et al., 2005; Leão et al., 2006). Presynaptically, there is little evidence as to whether the various steps in maturation are driven by activity, genetics, or a combination of both. Furthermore, we have no knowledge regarding the signalling cascades underlying these changes. Understanding the driving forces behind maturation provides a largely unexplored aspect of study at this synapse which hinges on, at a minimum, an equivalent understanding of the mature synapse that we currently have of the immature one. It is critical to understand the “target” or “final” state of the mature neuron in order to understand whether development has been altered in the absence of activity.

In the following chapters we have sought to better describe the fundamental release properties of the mature synapse, well into the third postnatal week, and compare it with those soon before the onset of sensory activity. In this pursuit, we have developed the ability to perform high-quality whole-cell recordings from the calyx of Held at ages up to P18, providing sufficient separation from the immature group (P8-12) to resolve subtle changes in presynaptic development. In particular, we have tried to gain fundamental insight into the subsynaptic arrangement of VGCCs and SVs during development, inaccessible to conventional imaging techniques at this synapse.

1.7.2 VGCC-SV Coupling, Synaptic Delay, & Short-Term Plasticity.

The nature of the spatial coupling between VGCCs and SVs has been debated throughout the literature and has prompted significant and long-standing debate as to how SVs are released. Understanding of this relationship, and whether it’s plastic, is crucial for understanding other aspects of presynaptic development. For example, developmental increases in the \( \text{Ca}^{2+} \) buffers calretinin and
parvalbumin have been observed at the calyx of Held (Felmy & Schneggenburger, 2004). Because these buffers have kinetics similar to EGTA, understanding the nature of VGCC-SV coupling at various developmental stages may alter interpretations regarding the role of these buffers. If VGCC-SV coupling is tight, then slow Ca\(^{2+}\) buffers such as these will have little impact on release evoked by single APs but will have implications for Ca\(^{2+}\) build-up during repetitive activity and STP. Conversely, if VGCC-SV coupling is loose, these buffers will tonically and significantly inhibit SV release even during single APs (Adler et al., 1991; Borst & Sakmann, 1996; Fedchyshyn & Wang, 2005). Thus, changes in VGCC-SV coupling can have a significant impact on the hypotheses drawn regarding the functional implication of developmental increases in Ca\(^{2+}\) buffer expression. In addition, the spatial relationship between VGCCs and SVs defines which steps in the AP-SV release cascade carry the most significant influence in determining \(P_r\). If we consider one extreme of possible VGCC-SV coupling modalities; one VGCC gates a single SV, then the release of an SV depends solely on the stochastic dependence of VGCC opening on the membrane voltage imposed by the AP. This effectively couples SV release completely to the APs effectiveness in opening each VGCC. In the alternative arrangement of many VGGCCs gating a single SV, release depends most significantly on the probability that an AP is effective at gating some number of VGCCs at the same instance, in the same general location. Thus, due to the high degree of non-linearity \((m)\) inherent in this arrangement \((I_{\text{EPSC}} \propto [I_{Ca}]^{m})\), \(P_r\) hinges most prominently on the occurrence of concurrent VGCC gating. The difference between these modalities imposes vastly different constraints on the dynamic range of the synapse and the means through which \(P_r\) can be modified (see section 1.2). At the calyx of Held, it is well accepted that many loosely-coupled VGCCs gate SV release in the immature synapse; however, this relationship was previously unknown in the mature synapse (Fedchyshyn & Wang, 2005). Our investigation addressing this question provokes re-examination of the synaptic mechanisms we presume to operate based on the data gained from immature synapses. The functional relevance of developmental changes in release modality is explored in the following chapters.

Another characteristic developmental change observed at the calyx of Held-MNTB synapse is a decrease in synaptic delay (Taschenberger & von Gersdorff, 2000); however, the mechanisms which lead to this change are currently unknown. Two primary issues have precluded the use of SD as a tool for furthering our understanding of the release process: first, SD changes are generally small and require accuracy in measurement beyond most current methods. Second, and as discussed above, few studies have investigated this property into functional maturation. The processes underlying SD may help us in the understanding of the SV release process and may place limits on the arrangement of its underlying components. For example, it has been established in this synapse that SV release kinetics depend primarily on the binding of Ca\(^{2+}\) ions to the Ca\(^{2+}\) sensor (Felmy et al., 2003), thus the dynamics of Ca\(^{2+}\)-domain establishment and spread are of primary importance in determining SD. A recent study by Wadel et al., (2007) demonstrated that a 2-fold difference in the [Ca\(^{2+}\)], experienced by the Ca\(^{2+}\) sensor can result in a 10-fold alteration in SV release kinetics. Considering the differing implications that loose and tight coupling has for the buffered diffusion of Ca\(^{2+}\) and the [Ca\(^{2+}\)] at the release sensor, it is likely that VGCC-SV coupling plays a role in explaining the developmental shortening of SD.
While it appears clear that VGCC-SV coupling has a number of implications for both the probability and kinetics of SV release, at a molecular level, we have little conclusive evidence as to which proteins define the separation between these two crucial components of release. The interactions between VGCCs and AZ proteins have been studied in a number of model and expression systems (see section 1.3 & 1.4). These data are often difficult to interpret, outside of their native system, due to the diversity that exists within the particular components of different AZs (i.e. different VGCC subtypes). One phenomenon that exemplifies this difficulty is the observation that N-type VGCCs are pruned out of the AZ with maturation at the calyx of Held and other synapses (Iwasaki et al., 2000). This observation begs two questions: first, why are tightly-coupled P/Q-type VGCCs specifically advantageous to the functioning of the mature synapse? Second, what molecular interactions change with development to initiate the switch is VGCC subtype in the AZ? Understanding first that these VGCCs are differentially targeted at the AZ during development provoke such questions and provide a system in which to parse the underlying mechanisms (see Cao et al., 2004). Understanding what final conformation the synapse is attaining following development allows for us to further hypothesize what components of the AZ must be present/absent in order to establish such an arrangement.

The calyx of Held-MNTB synapse has been particularly well used in understanding the mechanisms underlying various forms of STP such as STF and STD. As above, this has been particularly relevant for the immature synapse. While some descriptions of STP have been made in the mature synapse, presynaptic roles in STP have been largely inferred from data recorded from the postsynaptic MNTB neuron. Interestingly, the nature of VGCC-SV coupling has implications for rationalizing the types of STP observed at different developmental stages. Throughout the literature, modelling forms of STP, particularly STF, have required assumptions to be made regarding the nature of the Ca\(^{2+}\) sensor, which were or continue to be immeasurable (see section 1.6). These assumptions have arisen largely due to the lack of specific knowledge regarding the arrangement of VGCCs surrounding SVs and the unknown affinity of the Ca\(^{2+}\) sensor for Ca\(^{2+}\). If we consider the nanodomain modality, STF, by either bound model or residual Ca\(^{2+}\) model, cannot occur unless at least some SVs are releasable via multiple VGCCs. That is, they must experience subthreshold I\(_{\text{Ca}}\) in order to be facilitated, but paradoxically are also released upon single VGCC opening. Thus explaining STF with these models, assuming a given release modality, often require the inclusion of mechanisms outside the residual Ca\(^{2+}\) hypothesis to explain STF (i.e. lateral inhibition, second sensor models, AZ independence etc.). Whether these mechanisms are required to explain experimental observations hinges on a confident understanding of the release modality at the synapse.

Different synapses exhibit different native forms of STP (i.e. STF, STD, or a combination of both) during repetitive activity. These different synapses have been successfully modelled using different combinations of three Ca\(^{2+}\)-dependent mechanisms: facilitation, release site unavailability, and SV replenishment (Dittman et al., 2000). Herein, we demonstrate that differences in release modality, given identical input waveforms, can reproduce some of the diversity in STP patterns observed across different synapses. This suggests that distinct VGCC-SV coupling arrangements may underlie some of the diversity in STP observed throughout the CNS.
As the sophistication of synaptic modelling continues to advance it will need to foray into understanding how changes at the individual synapse level alter the functioning of the system in which it is contained. Thus, the requirement for deep understanding of the mature synapse, at a time when these systems are in fact active, will become an obvious necessity. Therefore, understanding the nature of VGCC-SV coupling allows for constraints to be placed on models aimed at explaining the observed patterns of STP and most likely influences those observed STP patterns also.

1.7.3 Focus of Contained Works.

In the subsequent chapters we have begun to address the above voids in calyx of Held-centric literature, with specific attention to the mature synapse, and have begun to extend that knowledge into fast synapses in general. We first asked whether the coupling between VGCCs and SVs changes with development, and whether this change is specific to a particular subtype of VGCC, in order to uncover this most fundamental property of the mature nerve terminal (see Chapter 3). We next asked whether changes in \( P_r \), initiated during typical synaptic activity (high-frequency trains), could elicit changes in SD that could be measured with sufficient accuracy to be used as an alternative tool in the study of synaptic transmission (see Chapter 4 & 5). We then employed our SD detection methodology (see section 2.4) to link SD to \( P_r \) through the coupling of VGCCs and SVs, confirming that the \([\text{Ca}^{2+}]\) profile experienced by the \( \text{Ca}^{2+} \) sensor is crucial in defining the kinetics of release.

We next proceeded to search for a protein(s) which underlies the distinct release modalities observed throughout development and came to Septin 5 as a likely candidate for fulfilling this role (see Chapter 5).

Finally, we asked how changes in release modality impact the nature of STP, in both synaptic strength and delay, observed and permissible at various stages of development. In this light we next proposed a minimal model in which VGCC-SV coupling is theoretically sufficient to explain much of the observed short-term plasticity at the calyx of Held-MNTB synapse (see Chapter 6).

The findings contained herein contribute in a novel way to our understanding of the subsynaptic development of this synapse, which elements may underlie it, and what impact is has on the transmission characteristics of this synapse. More fundamentally, we have taken steps to illustrate the fundamental state of the release complex at the mature calyx of Held-MNTB synapse. These data show that subtle presynaptic adjustments coalesce to significantly alter the properties of the synapse with development and force re-examination of dogma based on our more in-depth understanding of the immature synapse. Understanding how these changes occur, in this permissible synapse, will improve our understanding of how other fast central synapses acquire the character that justifies their name.
2. METHODOLOGY
2.1 SLICE PREPARATION

Mice were housed in the facility certified by the Canadian Council of Animal Care and used for this study according to a protocol approved by the Hospital for Sick Children Animal Care Committee. Brainstem slices were prepared from postnatal day 8 to postnatal day 18 (P8-18) mice (CD1/C57 black hybrid, unless otherwise noted) as previously described for rats (Forsythe, 1994). Following decapitation with a small guillotine, brains were immediately immersed into semi-frozen artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl (125), KCl (2.5), glucose (10), NaH₂PO₄ (1.25), Na-pyruvate (2), myo-inositol (2), ascorbic acid (0.5), NaHCO₃ (26), MgCl₂ (1), and CaCl₂ (2) at a pH of 7.3 when oxygenated (95% O₂ and 5% CO₂) followed by rapid dissection. Transverse slices of the auditory brainstem, containing the medial nucleus of the trapezoid body (MNTB), were cut at a thickness of 150-300 μm using a microtome (Leica VT100S, Leica Microsystems, Richmond Hill, ON) followed by incubation at 37°C for one hour prior to experimentation. For paired voltage-clamp recordings, thin slices (150-200 μm) were prepared slightly angled (~15 degree tilt away from the coronal plane) during slicing to minimize presynaptic axon length and reduce voltage-clamp errors. All experiments were performed at room temperature (RT, 20-22°C) unless otherwise noted. Experiments performed at near-mouse body temperature (35°C) were performed using an inline heater with feedback thermistor (Warner TC-324B, Hamden, CT). A list of pharmacological compounds used can be found in Table 2.1.

2.2 ELECTROPHYSIOLOGY

2.2.1 Equipment.

All electrophysiological recordings, both from postsynaptic MNTB neurons and presynaptic calyces of Held, were made with a dual-channel amplifier (MultiClamp 700A, Molecular Devices, Union City, CA) and digitized using a Digidata 1322A (Molecular Devices, Union City, CA). Afferent axon stimulation was performed by placing a platinum bipolar electrode near the midline of brainstem slices and inputting a brief (60 μs) potential difference across the termini. Stimulation was triggered through a Master-8 (A.M.P. Instruments, Jerusalem, Israel) stimulator and generated using an Iso-Flex Isolator (A.M.P. Instruments, Jerusalem, Israel). Patch electrodes were pulled using a PP-830 puller (Narashige USA, East Meadow, NY) to tip resistances of 4-6 MΩ and 2.5-3 MΩ for presynaptic and postsynaptic use respectively.

2.2.2 Intracellular Solutions.

Intracellular solution for postsynaptic voltage-clamp recordings contained (in mM) K-gluconate (97.5), CsCl (32.5), EGTA (5), HEPES (10), MgCl₂ (1), TEA (30) and QX314 (3) (pH=7.2). Intracellular solution (ICS) for presynaptic voltage-clamp recordings of Ca²⁺ currents contained (in mM) CsCl₂ (110), HEPES (40), EGTA (0.5), MgCl₂ (1), ATP (2), GTP (0.5), Phosphocreatine (12), TEA (20), and K⁺-glutamate (3) (pH adjusted to 7.3 with CsOH). For presynaptic current-clamp and presynaptic cell-attached voltage-clamp recordings, pipettes were filled with a solution containing (in mM) K-gluconate
Intra-terminal glutamate concentration was determined in experiments (not shown) in which differing amounts of K⁺-glutamate (1-5mM, 1mM increments) was added to the presynaptic intracellular solution and afferent-evoked I_{EPSC} were generated prior to and following presynaptic dialysis. We noted potentiation of I_{EPSC} when [Glutamate] was 4mM or higher and I_{EPSC} rundown when [Glutamate] was 2mM or lower. Supplementation of 3mM K⁺-Glutamate resulted in no change in I_{EPSC} for the permissible duration of recordings (>45min.), including bouts of high-frequency stimulation. Thus, 3mM Glutamate was assumed to be the endogenous concentration and was used in presynaptic ICS in all whole-cell recordings herein. A list of pharmacological compounds used can be found in Table 2.1.

2.2.3 Extracellular Solutions.

aCSF was supplemented with bicuculline (10 μM) and strychnine (1 μM) to block inhibitory inputs during recording, [Ca^{2+}]_o was adjusted throughout as noted in the text. For recording of presynaptic Ca^{2+} currents, tetrodotoxin (TTX, 0.5μM), tetraethylammonium (TEA, 10 mM) and 4-aminopyridine (4-AP, 0.3 mM) were added to block Na⁺ and K⁺ channels respectively. For experiments with ω-agatoxin-IVA (ATX) and ω-conotoxin-GVIA (CTX), slices were first perfused with aCSF containing cytochrome C (500 µg/ml) before the desirable amount of either toxin was added (Iwasaki & Takahashi, 1998; Wu et al., 1999). For experiments in which cyclothiazide (CTZ) was used, it was dissolved in dimethyl sulfoxide (DMSO) and then added to aCSF, to a final concentration of 50μM and 0.05% DMSO. A list of pharmacological compounds used can be found in Table 2.1.

2.2.4 General Electrophysiological Recording.

For paired voltage-clamp recordings, presynaptic and postsynaptic series resistances were 6-12MΩ (<10MΩ in the majority of recordings) and 4-10MΩ respectively and compensated to 90% with cells showing higher resistances being omitted from analysis. The holding potential was -70 or -80mV and -60mV for presynaptic terminals and postsynaptic neurons respectively. Presynaptic Ca^{2+} currents (I_Ca) were evoked by the various voltage-command protocols, as indicated in the text, with leak subtraction performed with the on-line P/4 protocol. All presynaptic voltage-command waveforms were generated in the pCLAMP 8.2 amplifier interface (Molecular Devices, Union City, CA).

2.2.5 Use of Recorded Waveforms as Presynaptic Voltage-Commands.

For experiments in which real APs, or AP trains, were used as presynaptic voltage-command waveforms, we first recorded the desired waveform in current-clamp configuration in response to afferent stimulation. Waveforms were sampled at 50KHz and filtered online at 4KHz. After manually removing the stimulation artefacts preceding the APs, the digitized voltage values were normalized, scaled and fed back into the amplifier as a stimulus file (.atf), through the pCLAMP 8.2 amplifier interface, at the same frequency as their acquisition.
2.2.6 Pharmacology.

All reagents were purchased from Sigma-Aldrich Canada (Oakville, ON), Tocris (Ellisville, MI) and Alomone Labs (Jerusalem, Israel). A list of pharmacological compounds used can be found in Table 2.1.

2.3 DATA ANALYSIS

2.3.1 General Data Handling.

Data were acquired on-line, filtered at 4KHz, digitized at 50KHz and analysed off-line using the pCLAMP 8.2/9.2 software package (Molecular Devices, Union City, CA), Excel 2000 (Microsoft, Redmond, WA), Mini Analysis Program (Synaptosoft, Decatur, GA), and Maple 7/9.5 (Maplesoft, Waterloo, ON). Statistical tests of significance were two-tailed, unpaired Student’s t-tests assuming unequal variances with a p-value cut-off of <0.05. For some data, Kolmogorov-Smirnov (K-S) 2-sample tests were used to determine significance, when data distributions deviated from normality, with a p-value cut-off of <0.05. Data were expressed as the mean ± standard error (S.E.) from a population of synapses (n).

2.4 CHAPTER 3 SPECIFIC METHODOLOGIES

2.4.1 Electrophysiology.

For presynaptic current-clamp recordings, the intracellular solution was supplemented with either 10mM EGTA or 1mM BAPTA, as indicated in the text. Presynaptic intracellular solution for voltage-clamp recordings was supplemented with 10mM EGTA for certain experiments as noted in the text (see Sakmann, 2006). For AP_L (Action Potential-like: -70mV to +60mV steps; 100μs incremental increases in repolarization time) and AP_D (Action Potential-duration: -70mV to +60mV steps; 100μs incremental increases in plateau time) paradigms, on- and off-time of command voltage were set at 200μs that resembled rise and repolarization time courses of action potentials and also minimized artefacts of leak subtraction.

2.4.2 Data Analysis.

For cooperativity analysis, peak amplitudes or area integrals for both Ca^{2+} currents and EPSCs were detected using analysis windows that began at the onset of I_{Ca} and I_{EPSC}, and ended at the points that contain entire area of I_{Ca} tail current and the area up to the 10% decay point of I_{EPSC} peak amplitude. Current peaks and integrals were normalized to the maximal current (100%). The logarithms of peak or area values were then plotted against each other on linear axes and normalized to their respective maximal currents, within their linear range according to the following equation, using either current peaks or integrals:
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Chemical Name</th>
<th>Target/Action</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ω-Agatoxin-IVA</td>
<td></td>
<td>P/Q-Type VGCC Blocker</td>
<td>Alomone Labs</td>
</tr>
<tr>
<td>ω-Conotoxin-GVIA</td>
<td></td>
<td>N-Type VGCC Blocker</td>
<td>Alomone Labs</td>
</tr>
<tr>
<td>4-aminopyridine (4-AP)</td>
<td>4-Pyridinamine</td>
<td>K⁺ channel antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrasodium salt</td>
<td>Ca²⁺ chelator</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>(-)-Bicuculline methiodide</td>
<td>GABA&lt;sub&gt;β&lt;/sub&gt; receptor antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>Baclofen</td>
<td>(R)-4-Amino-3-(4-chlorophenyl) butanoic acid</td>
<td>GABA&lt;sub&gt;β&lt;/sub&gt; receptor agonist</td>
<td>Tocris</td>
</tr>
<tr>
<td>Cesium Chloride</td>
<td>CsCl</td>
<td>Non-selective K⁺ channel blocker</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cyclothiazide</td>
<td>6-Chloro-3,4-dihydro-3-(5-norbornen-2-yl)-2H-1,2,4-benzothiazidiazine-7-sulfonamide-1,1-dioxide</td>
<td>Inhibits AMPAR desensitization</td>
<td>Tocris</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td></td>
<td>Prevents non-specific binding of toxins.</td>
<td>Sigma</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt</td>
<td>Ca²⁺ chelator</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.1: List of Pharmacological Compounds.
<table>
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<th>Chemical Name/Synonym</th>
<th>Target/Action</th>
<th>Supplier</th>
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</thead>
<tbody>
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<td>EGTA-AM</td>
<td>Ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetoxyethyl Ester</td>
<td>Membrane-permeable Ca(^{2+}) chelator</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Glutamate</td>
<td>(S)-2-Aminopentanedioic acid</td>
<td>Excitatory amino acid neurotransmitter</td>
<td>Sigma</td>
</tr>
<tr>
<td>Kynurenic Acid</td>
<td>4-Hydroxyquinoline-2-carboxylic acid</td>
<td>Non-selective Glutamate Receptor Antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>QX 314</td>
<td>N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide</td>
<td>Intracellular blocker of voltage-activated Na(^{+}) channels</td>
<td>Tocris</td>
</tr>
<tr>
<td>Strychnine</td>
<td>(-)-Strychnine</td>
<td>Glycine receptor antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium bromide</td>
<td>Voltage-gated K(^{+}) channel blocker</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tetrodotoxin (TTX)</td>
<td>Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol citrate</td>
<td>Voltage-gated Na(^{+}) channel blocker</td>
<td>Alomone Labs</td>
</tr>
</tbody>
</table>

Table 2.1: List of pharmacological compounds.
\[
\log \left( \frac{I_{Ca}}{\text{max } I_{Ca}} \times 100 \right) \quad \text{vs.} \quad \log \left( \frac{I_{EPSC}}{\text{max } I_{EPSC}} \times 100 \right)
\]

Least-squares linear regression was performed using Excel 2000 (Microsoft, Redmond, WA) to obtain the slope value defined as \( m \), the cooperativity. The \( m \) values were virtually identical when amplitude or area integrals were chosen for analysis but only results based on area integrals are presented. As only a subset of data situated in the initial linear range of the \( \log[I_{Ca}] \) vs. \( \log[I_{EPSC}] \) plot (i.e. low input vs. output) was used to determine this parameter for each synapse, postsynaptic receptor saturation unlikely played a significant role in \( m \). The amplitude of \( I_{EPSC} \) included for analysis were usually well below the maximum of the synapses dynamic range.

2.5 CHAPTER 4 SPECIFIC METHODOLOGIES

2.5.1 Electrophysiology.

For experiments in which EGTA-AM was used, slices were immersed, following incubation, in aCSF containing 50 \( \mu \)M EGTA-AM for 30 min. at room temperature preceding experimentation. In measuring synaptic delay, presynaptic cell-attached recordings (voltage-clamp mode) were performed to facilitate long-lasting, high-accuracy recordings of presynaptic APs and to leave the presynaptic terminal undisturbed. The real AP trains, used to evoke Ca\(^{2+}\) currents from voltage clamped calyces, were recorded from a P15 calyx as described in section 2.2.5. Baclofen was dissolved directly into aCSF to a final concentration of 50\( \mu \)M as noted in the text.

2.5.2 Data Analysis.

Component neurotransmission delays were defined as follows; Conduction delay (CD) (Fig. 4.1A, segment 1) was defined as the delay between the peak of the stimulation artefact and the negative deflection of the presynaptic volley, which represents the maximum rate of depolarization of the presynaptic AP (Sabatini & Regehr; 1997; Yang & Wang, 2006). Synaptic delay (SD) (Fig. 4.1A, segment 2) was defined as the delay between the downward deflection of the presynaptic volley and the onset of the \( I_{EPSC} \). Response-rise delay (RRD) (Fig. 4.1A, segment 3) was defined as the delay between the onset of \( I_{EPSC} \) and its peak. Finally, transmission delay (TD) (Fig. 4.1A, segment 4) was defined as the sum of the delays above. Fitting for the determination of the \( I_{EPSC} \) onset was performed in Clampfit 8.2/9.2 (Molecular Devices, Union City, CA) using a Levenberg-Marquardt search algorithm minimizing the sum of squared errors for the chosen function (Fig. 4.1C; also see eqn. (13) in section 2.5.7). Linear fitting, for error analysis and delay/variance progression, was also performed using a least squares minimization. Baseline averages were sampled from a 10ms portion of the recording preceding stimulation. Envelope plots (Fig. 4.4, 4.5, 4.7, & 9.1.1) show individual delay values, as a function of event number, for 30 repeated trains at specified frequencies. Progression of delay was calculated as
the slope of the least-squares linear fit through the 900 points displayed on each envelope plot, and is reported as $\mu s \cdot \text{event}^{-1}$. Variance of the reported delays was calculated for each event and plotted against event number (data not shown), a linear least-squares fit was then used to determine the progression of the variance, reported as the slope in $\mu s^2 \cdot \text{event}^{-1}$. The recovery time constants for both $I_{\text{EPSC}}$ area and each delay component (Fig. 4.9) were fitted with one- or two-term standard exponential equations, with their asymptote fixed at 100%, and using a Levenberg-Marquardt search algorithm minimizing the sum of squared errors. A summary of significance tests between all tested stimulation frequencies and experimental groups is provided in Table 4.3. Significance is assessed for delay (blue) and variance (green), within the same comparison matrix, for each delay component.

2.5.3 Determining the EPSC Onset – Intersection Method.

The “Intersection Method” defines the $I_{\text{EPSC}}$ onset as the intersection point of two lines fit to the experimental data. The first is a horizontal line fit to the baseline region preceding the onset of the $I_{\text{EPSC}}$, the choice of what section of $I_{\text{EPSC}}$ constitutes the baseline being arbitrary. The second is fit to the rising-phase of the $I_{\text{EPSC}}$ which is defined as an arbitrary range, usually either 10-90% or 20-80% of the $I_{\text{EPSC}}$ peak (Fig. 2.1B). The equations of the lines are given as:

\[ I = I_h \quad (1) \]
\[ I = m_f t + b_f \quad (2) \]

Where $I_h$ is the baseline current, $m_f$ is the slope of the line fitted to the $I_{\text{EPSC}}$ rising-phase, and $b_f$ is the $I$-intercept of the line fitted to the $I_{\text{EPSC}}$ rising phase. Setting equations (1) and (2) equal allows for the determination of $t$ at the intersection point which is the defined $I_{\text{EPSC}}$ onset using this method.

2.5.4 Intersection Method – Sources of Error.

While this method is algebraically simple, it has sources of error in both equations (1) & (2) which affect its overall accuracy. Equation (2) is subject to error in both of its parameters, $m_f$ and $b_f$, which depend on deviations of the source data ($I_{\text{EPSC}}$ rising-phase) from linearity. The sum of squared errors is minimized when the parameters are given by:

\[ m_f = \frac{N \left( \sum (t_i I_i) - (\sum t_i)(\sum I_i) \right)}{N \left( \sum (t_i)^2 - (\sum t_i)^2 \right)} \quad (3) \]
\[ b_f = \frac{\sum I_i - m_f \sum t_i}{N} \quad (4) \]

With standard deviations given by;
\[ S_m = \sqrt{\frac{N s_i^2}{N \sum t_i^2 - (\sum t_i)^2}} \]  

(5)

\[ S_h = S_m \sqrt{\frac{\sum t_i^2}{N}} \]  

(6)

Where \( s_i \) is the residual standard deviation. Then, the standard error in each parameter can be calculated depending on the confidence interval chosen (95% for all analyses). The error in the \( I_{\text{EPSC}} \) onset is then the range between \( t \)-intercepts calculated using the upper and lower error limits of the fitted slope (Fig. 2.1B, left panels).

\[ I - I_{\text{mid}} = (m_f + S_m)(t - t_{\text{mid}}) \]  

(7)

\[ I - I_{\text{mid}} = (m_f - S_m)(t - t_{\text{mid}}) \]  

(8)

Where \((I_{\text{mid}}, t_{\text{mid}})\) is the midpoint of the fitted line and are common with both boundary lines above (equations (2),(7), & (8)). Setting \( I = I_h \), and subtracting (7) and (8) yields the standard error in the estimation of the \( I_{\text{EPSC}} \) onset. It can also be shown that, by setting \( t = 0 \) in (2) and (7) or (8), the difference in the \( I \)-intercept is equal to (6).

When we consider that equation (1) also carries error from the averaging of \( I_h \), the error in the \( I_{\text{EPSC}} \) onset is increased further. Determination of the error in \( I_h \) (\( \Delta I_h \)) involves averaging over a region in which \( I \) fluctuates normally (noise) around \( I_h \). Thus the error in \( I_h \) is the standard error of the mean, where \( \sigma \) is the standard deviation.

\[ \Delta I_h = \frac{\sigma}{\sqrt{N}} \]  

(9)

Although this error only occurs in the \( I \)-dimension, it affects the accuracy of the \( I_{\text{EPSC}} \) onset through its effect on the intersection of (1) with (2). The magnitude of this error is dependent on the level of “noise” contained within the chosen baseline region and the degree to which it is stationary. Setting \( I = I_h - \Delta I_h \) in (8) or \( I = I_h + \Delta I_h \) in (9) increases both the upper and lower limit of \( t \) beyond \( S_m \) (Fig. 2.1B, rightmost panel). Thus equations (7) and (8) become;

\[ (I + \Delta I_h) - I_{\text{mid}} = (m_f + S_m)(t - t_{\text{mid}}) \]  

(10)

\[ (I - \Delta I_h) - I_{\text{mid}} = (m_f - S_m)(t - t_{\text{mid}}) \]  

(11)

Which, when solved for \( t \), represent the upper and lower errors for the \( I_{\text{EPSC}} \) onset with error in (1) and (2) considered.
Figure 2.1: Quantitative Assessment of Three Analytical Methods for Determination of the Onset of EPSCs.

(A) Example EPSC (grey line, left panel), with fitted Boltzmann equation (black line), and dotted lines highlighting the region used for the curve fitting process. The fitted function (grey line) and the 4th derivative (black line), aligned in time, are shown (right panel) with solutions to the 4th derivative given as vertical dotted lines (t₁, t₂, t₃). (B) Same trace as in A analyzed with the “Linear Intersection Method”. The EPSC onset is determined by the intersection point of the two black lines, with the upper and lower error limits shown by dotted lines (left panel). The boxed region is magnified (right panel) to show the shift in the calculated EPSC onset time with and without the inclusion of error in the horizontal baseline. Nomenclature refers to the Methodology section 2.5. (C) Same trace as in A analyzed with the “Threshold Method”. The black line in the left panel indicates 10% of the EPSC peak. Magnification of the boxed region is shown (right panel) to illustrate the error inherent due to digitization. (D) Cumulative histograms of the error distributions in the determination of the EPSC onset from the first (grey line) and last (black line) EPSCs in response to train stimuli (200Hz, 150ms), using the linear intersection (left panel) and maximal curvature methods (right panel).
2.5.5 Determining the EPSC Onset – Threshold Method.

The “Threshold Method” defines the $I_{\text{EPSC}}$ onset as the time point at which the $I_{\text{EPSC}}$ is an arbitrary percentage of its maximum, usually 10% (Fig. 2.1C).

2.5.6 Threshold Method – Sources of Error.

The accuracy of the method is determined by the rate at which the analog current signal is digitized. The temporal spacing between samples [i.e. $(l_1,t_1)$ & $(l_2,t_2)$, Fig. 2.1C, rightmost panel] creates an interpolated region in which the 10% point will almost always fall. The 10% point must then be taken as the known digitized point ($l_1$ or $l_2$) closest to the calculated value ($I_{\text{actual}}$). Choosing either of these points also means choosing the corresponding time point ($t_1$ or $t_2$) as the $I_{\text{EPSC}}$ onset. Since the probability of $I_{\text{actual}}$ occurring at any point between $t_1$ and $t_2$ is uniform, the error in the $I_{\text{EPSC}}$ onset is:

$$\Delta t_{\text{onset}} = \pm \frac{S_{\text{dig}}}{2}$$

Where $S_{\text{dig}}$ is the inter-sample interval of the digitizer. Thus, the error in the determination of the $I_{\text{EPSC}}$ onset is uniform using this method.

2.5.7 Determining the EPSC Onset – Maximum Curvature Method.

The “Maximum Curvature” Method defines the $I_{\text{EPSC}}$ onset as the point of maximum curvature occurring during the rising-phase of the $I_{\text{EPSC}}$. To determine this point, an equation in the form of the Boltzmann function is used (Fig. 2.1A).

$$I(t) = \frac{I_{\text{max}}}{1 + \exp\left(\frac{(t_{\text{mid}} - t)}{t_c}\right)} + C$$

(13)

Since we are concerned only with the initial portion of the $I_{\text{EPSC}}$, the fitted region can be chosen such that it maximizes the fit quality in the region of $I_{\text{EPSC}}$ onset (solid line in Fig. 2.1A, left panel, within fitted region indicated by dashed lines). This sacrifices accuracy in other regions of the fitted curve (red dotted line), which are inconsequential to the determination of the $I_{\text{EPSC}}$ onset. With the parameters determined, we determine the point of maximum curvature by solving the 4$\text{th}$ derivative of (13) and set it equal to 0. Evaluation of the 4$\text{th}$ derivative gives three solutions (Figure 2.1B, bottom panel):

$$t_1 = t_{\text{mid}} - \ln\left(5 + 2\sqrt{6}\right) t_c$$

(14)

$$t_2 = t_{\text{mid}}$$

(15)

$$t_3 = t_{\text{mid}} - \ln\left(5 - 2\sqrt{6}\right) t_c$$

(16)
Where the solution that occurs earliest in time is defined as the $I_{EPSC}$ onset.

### 2.5.8 Maximum Curvature Method – Sources of Error.

The accuracy of the “Maximum Curvature Method” is determined by the accuracy in the determination of $t_{\text{mid}}$ and $t_{c}$ through the fitting process (Levenberg-Marquardt with least-squares minimization). Here, the error is determined from the evaluation of a covariance matrix of the general form;

$$\text{cov}(X_i, X_j) = E[(X_i - \mu_i)(X_j - \mu_j)]$$  \hspace{1cm} (17)

Where $\mu$ is the mean and $E$ is the matrix of expected values for $X$. In the condition where $i=j$, (17) reduces to;

$$\text{cov}(X_i, X_j) = E[(X_i - \mu)^2] = \text{var}(X_i)$$  \hspace{1cm} (18)

Which is the variance of the parameter matrix $X_i$. Following standard error propagation rules, we can evaluate $t_1$ or $t_3$, whichever occurs earlier in time, and summate the standard error returned from (18) to determine the overall error in the $I_{EPSC}$ onset.

### 2.5.9 Example Error Determination – Intersection Method.

Choosing the thirty example traces shown in Fig. 4.2A, we determined the accuracy of each $I_{EPSC}$ onset detection method for the first and final event in the train (200Hz, 150ms). The maximal $I_{EPSC}$ amplitude was determined for each event from which the 10% and 90% points were also determined. The digitized points nearest the 10/90 values were then chosen as the upper and lower range, respectively, for the linear least-squares fit to the rising-phase. A 300\,$\mu$s region preceding the $I_{EPSC}$ and following the stimulation artefact was chosen as the baseline and used to determine $I_h$. Regression analysis was performed (as above) for data points within the 10% and 90% range, from which the equation of the fitted line and the error in its parameters were returned. The range of $t$-intercepts was then calculated with and without error in $I_h$ for each of the traces. The analysis was then repeated for the final (30\,$^\text{th}$) event in each sweep.

In the absence of error in $I_h$, the error in the determination of the $I_{EPSC}$ onset was $0.0044\pm0.0002\,$ms for the first event and $0.014\pm0.005\,$ms for the final event. The increase in error of the final event can be attributed directly to the increased noise inherent in the rising-phase of small depressed $I_{EPSC}$, which occur late in trains where release synchronization has broken down. Under these conditions, the error in the slope of the rising-phase increases ($S_m$), increasing the error in the $t$-intercept.

When the error in $I_h$ was calculated it had an average value of $1.58\pm0.07\,$pA and $2.0\pm0.1\,$pA for first and last events respectively. Adding this to the previous error, we observe a small increase in
average error to 0.0046±0.0005ms for the first events and a more significant increase in the error of the final events to 0.016±0.005ms. That is, as the slope of the line fitted to the rising-phase decreases, the length of time travelled between $I_h$ and $\Delta I_h$ increases, resulting in the increased error. The distributions of errors in both the first and final events are compared as a cumulative histogram in Fig. 2.1D (left panel).

### 2.5.10 Example Error Determination – Threshold Method.

Error determination for the “Threshold Method” is directly related to digitizing speed. For the experiment in question, and those throughout, the sampling rate was 50kHz producing a time-gap between acquired points of 0.02ms. Using (12), a consistent error of 0.01ms is inherent in the time point reported near 10% of the maximal $I_{EPSC}$.

### 2.5.11 Example Error Determination – Maximum Curvature Method.

The error in the determination of the $I_{EPSC}$ onset, using the “Maximum Curvature Method”, results from the propagation of error contained the $t_{mid}$ and $t_c$ parameters. For the first events the average error was 0.0035±0.0002ms with only a modest increase for the final events being 0.0051±0.0005ms on average. The distributions of the errors in both the first and final events are compared as a cumulative histogram in Fig. 2.1D (right panel). In summary, the maximum curvature method can detect the $I_{EPSC}$ onset with accuracy as much as 2 orders of magnitude better than either of the other methods.

### 2.5.12 Special Cases & Methodological Bias.

For large, fast $I_{EPSC}$, both the “Intersection Method” and the “Maximum Curvature Method” allow for determination of time points with microsecond accuracy. The “Threshold Method” is approximately one order of magnitude less accurate for the first event but is of similar accuracy later in $I_{EPSC}$ trains, when the accuracy of the “Linear Intersection Method” degrades significantly. However, the “Maximum Curvature Method” maintains microsecond accuracy independent of the stimulus number within the train.

Independent of their accuracies, both the “Intersection Method” and “Threshold Method” depend on parameters that can bias their determination of the $I_{EPSC}$ onset. In particular, they depend on the slope of the $I_{EPSC}$ rising-phase. A slowing in the kinetics of the $I_{EPSC}$ rise often results during high frequency stimulation due to desynchronization of release events or receptor desensitization towards the end of stimulation trains. To illustrate this bias, we chose two $I_{EPSC}$ of equal amplitudes but differing rising-phase slopes and compared the analytic solutions obtained with each of the three methods (Fig. 4.2C,D).

For the “Intersection Method”, as the slope of the $I_{EPSC}$ rise phase decreases, the intersection point is pushed back in time ($t_{black}$ vs. $t_{gray}$, Fig. 4.2C, left panel) despite near identical $I_{EPSC}$ onsets. Thus, this method cannot be used to compare $I_{EPSC}$ onset when $m_f$ changes, as it would mask potential increases in SD in traces with mild rising-phase slopes.

In contrast, the “Threshold Method” biases forward in time as the rising-phase slope decreases ($t_{black}$ vs. $t_{gray}$, Fig. 4.2C, right panel). This is due to the fact that it takes more time to reach 10% of the maximal $I_{EPSC}$ when the rising-phase slope declines, exacerbating SD in traces with mild $m_f$. Use of this
method, to evaluate the $I_{EPSC}$ onset, should then also be restricted to conditions in which the $I_{EPSC}$ rise-phase slope does not change.

The “Maximum Curvature Method” depends only on the shape of the $I_{EPSC}$, in the region of its onset, to establish the parameters that will be used to evaluate the 4th derivative. Taking the 4th derivative of (13) we get;

$$
\frac{d^4 I}{dt^4} = \frac{I_{max} \exp\left(\frac{t_{mid} - t}{t_c}\right) \left(\exp\left(3 \frac{t_{mid} - t}{t_c}\right) - \exp\left(2 \frac{t_{mid} - t}{t_c}\right) + \exp\left(\frac{t_{mid} - t}{t_c}\right) - 1\right)}{\left(1 + \exp\left(\frac{t_{mid} - t}{t_c}\right)\right)^5 t_c^4}
$$

(19)

and the 1st derivative, representative of the slope of $I(t)$;

$$
\frac{dI}{dt} = \frac{I_{max} \exp\left(\frac{t_{mid} - t}{t_c}\right)}{\left(1 + \exp\left(\frac{t_{mid} - t}{t_c}\right)\right)^2 t_c}
$$

(20)

which can be separated from (19). Setting (20) = $\beta$ we have;

$$
\frac{dI}{dt} = \frac{\beta \exp\left(\frac{3 t_{mid} - t}{t_c}\right) - \exp\left(\frac{2 t_{mid} - t}{t_c}\right) + \exp\left(\frac{t_{mid} - t}{t_c}\right) - 1}{\left(1 + \exp\left(\frac{t_{mid} - t}{t_c}\right)\right)^3 t_c^3}
$$

(21)

Setting (21) = 0 and solving for $t$ we get solutions (14-16) which are independent of $\beta$ and thus the slope of the $I_{EPSC}$.

This analysis suggests that the “Maximum Curvature Method” can be used to evaluate the $I_{EPSC}$ onset under conditions where the rising-phase of the $I_{EPSC}$ changes. Furthermore, determination of the $I_{EPSC}$ onset can be made with higher accuracy than by either of the other determination methods.
2.6 CHAPTER 5 SPECIFIC METHODOLOGIES

2.6.1 Electrophysiology.

Single real APs were from an immature (P10) calyx, in response to afferent stimulation, as described in section 2.2.5. For EGTA experiments, presynaptic Ca$^{2+}$ current recording solution was supplemented with 10mM EGTA. 0.2mM tetraethyl ammonium (TEA) and 1mM kynurenic acid (KYN) were dissolved directly in aCSF and applied extracellularly as noted throughout the text.

2.6.2 Data Analysis.

Synaptic Delay was defined and calculated as described in section 2.5 using the peak of presynaptic I$_{Ca}$ as an initial timing reference point rather than the AP. For slope factor analysis, normalized I$_{Ca}$ density was plotted against normalized SD. This relationship was well described by a linear function, the slope of which was defined as "slope factor" (s). For RRP recovery analysis recovery curves were fit with biphasic exponential equations with their horizontal asymptote forced to 100%. RRP current was evaluated using the method of Schneggenburger et al. 1999. Briefly, a plot of cumulative EPSC peak amplitude vs. event was constructed. A linear fit was performed to the points which provided the highest $R^2$ value and extrapolated to the y-axis to determine the total RRP current. Release fraction was calculated as the current density of the initial event in a stimulus train divided by the total current density from the same train.

2.6.3 Electron Microscopy & SV Separation distance Quantification.

Brainstem slices were prepared as described in section 2.1 but cut at a thickness of 300µm. Following recovery (1hr), slices were placed in fixative (2.5% gluteraldehyde in 0.1M cacodylate buffer, pH 7.4) for two hours. Slices were then washed in 0.1M cacodylate buffer four times at 10min. intervals. Sections were postfixed in osmium tetroxide (1% in 0.1M cacodylate buffer), saturated in uranyl acetate (1% in water) for one hour, and washed four times for ten minutes in water. Following washing sections were dehydrated and flat embedded in Durcapan resin (Fluka, EMS - Hatfield PA). All tEM fixation and subsequent preparation performed by J. Aitoubah.

Serial ultra-thin sections were cut at the University of Toronto electron microscopy facility using an RMC MT7000 ultramicrotome to a thickness of 80nm. Sections were collected onto high-transmission copper grids (50-mesh) and subsequently contrasted with 5% aqueous uranyl acetate. Micrographs were collected with an AT digital camera (6MP) inside a Hitachi 7000 transmission electron microscope at a filament voltage of 75kV.

SV-AZ separation was evaluated by measuring the distance between the closest points on the vesicle membrane and the visually identifiable AZ membrane. SVs within 2 vesicle diameters (~100nm) of the AZ were measured with those separated by less than 10nm being considered physically “docked”. Normalized SV separation distributions were evaluated using OpenLab software (OpenLab, Coventry UK) and plotted using Microsoft Excel 2000. Approximately 1600 SV separation measurements were taken from age-matched calyces for the immature (P9) and mature (P17) experimental groups from a
minimum of 5 calyces per group, similarly for immature and mature Sept5\(^{-/-}\) and Sept5\(^{+/+}\) synapses as noted throughout the text. SV separation distances were grouped into 2.5nM bins and plotted as noted throughout the text. All analyses were performed blinded.

2.6.4 Immunoflorescence.

Brainstem slices were prepared, as in section 2.1, at a thickness of 200\(\mu\)m, from Sept5\(^{+/+}\) and Sept5\(^{-/-}\) mice (P12). Following recovery for 1 hour at 37\(^\circ\)C, slices were fixed in 4\% paraformaldehyde in PBS for 30min. at room temperature. Slices were then washed with PBS for 30min. and permeabilized with 0.2\% Triton X-100 for 30min., followed by a second wash with PBS for 30min. A Mouse-on-Mouse (MOM) mouse IgG blocking reagent (Vecta Laboratories, Burlingame, CA) was subsequently used to block for 1hr. Slices were washed with PBS for 30min. and then incubated with MOM protein diluent (Vecta Laboratories, Burlingame, CA) for 30min. Brainstem slices were incubated overnight at 4\(^\circ\)C with an antibody to Sept5 (SP20) in MOM protein diluent at a concentration of 1:10. The following day, the primary antibody was removed by washing slices with PBS for 30min., followed by incubation with a goat anti-mouse secondary antibody conjugated to Alexa 488 (Jackson ImmunoResearch, Westgrove, PA) in MOM protein diluent at a concentration of 1:500 for 1hr. Slices were then washed with PBS for 30min. and incubated with an antibody to VAMPs 1-3 (Synaptic Systems, Goettingen, Germany) in MOM protein diluent at a concentration of 1:1000 overnight at 4\(^\circ\)C. The following day, the primary antibody was removed by washing slices with PBS for 30min. The slices were then incubated with a donkey antirabbit secondary antibody conjugated to Cy3 (Jackson ImmunoResearch, Westgrove, PA) in MOM protein diluent at a concentration of 1:500 for 1hr., followed by washing in PBS for 30min., and labelled for DNA using Hoecsht 33342 (Invitrogen, Burlington, ON, CAN) dissolved in PBS at 1:5000 for 30 min. Following a final wash with PBS, the slices were mounted onto glass slides with Dako mounting medium (Glostrup, Denmark). Images were taken with a Zeiss LSM510 Multiphoton Laser Scanning Confocal Microscope (Zeiss, Thornwood, New York) and processed with Adobe Photoshop and Adobe Illustrator software. Preparation of tissue was performed by MJ Fedchyshyn with subsequent preparation of tissue for confocal microscopy and imaging performed by CW Tsang.

2.7 CHAPTER 6 SPECIFIC METHODOLOGIES

2.7.1 Electrophysiology.

Immature AP trains (AP\(_{I}\)) were recorded in current-clamp from a P10 calyx and mature AP trains (AP\(_{M}\) & AP\(_{35}\)) were recorded from a P16 calyx, at room temperature and at 35\(^\circ\)C respectively, in response to afferent stimulation (200Hz, 200ms).
2.7.2 Data Analysis.

Synaptic Delay was defined and calculated as described in section 2.5 using the peak of $I_{\text{Ca}}$ as an initial timing reference point rather than the AP. The early and late progression rates of SD ($s_E$ & $s_L$) were evaluated as the slope of the least-squares linear fit to the first 5 events or the 6th to 20th events. Facilitation and depression indices were calculated by following formulas;

$$FI = \left[ 100 - \left( \frac{EPSC_1}{EPSC_{\text{MAX}}} \times 100 \right) \right]$$

$$DI = \left[ 100 - \left( \frac{EPSC_{40}}{EPSC_{\text{MAX}}} \times 100 \right) \right]$$
3. DEVELOPMENTAL TRANSFORMATION OF THE RELEASE MODALITY AT THE CALYX OF HELD SYNAPSE
3.1 INTRODUCTION

In developing central synapses, synaptic modifications likely take place concurrently in both presynaptic terminals and postsynaptic neurons (Li & Sheng, 2003). Although remarkable progress has been made in understanding the organization of postsynaptic receptors and their scaffolding structures (Li & Sheng, 2003; McGee & Bredt, 2003), little is known about changes in the spatial coupling of voltage-gated Ca\textsuperscript{2+} channels (VGCCs) to vesicular release of neurotransmitter in the nerve terminal. Inaccessibility to tiny nerve terminals of typical central synapses has primarily precluded direct electrophysiological analysis of these two most critical elements engaged in transmitter release. As a result of the large size of presynaptic calyces, the giant calyx of Held-MNTB synapse in the auditory brainstem permits simultaneous recordings from both presynaptic and postsynaptic elements (Borst et al., 1995; Takahashi et al., 1996), providing an excellent model for studying the mechanisms of fast synaptic transmission. This synapse is an axosomatic glutamatergic synapse critically involved in the sound localization circuit, in which the temporal fidelity of synaptic transmission, even at high frequencies (up to several hundred Hz), must be preserved for the auditory brain to compute interaural timing differences and ultimately determine the audible source in space (Trussell, 1999). Within the first two postnatal weeks, profound adaptations in morphological structure and biophysical properties of a variety of ion channels converge to facilitate the capability of transmission at high rates across this synapse (von Gersdorff & Borst, 2002). Although presynaptic release is one of the most important elements in determining synaptic efficacy, it remains primarily unknown how the calyceal terminal develops its ability to precisely control quantal output during this critical period of development.

The relationship between Ca\textsuperscript{2+} influx and quantal output (Q) at this and other synapses can be approximated by the following equation: 

\[ Q = [\text{Ca}^{2+}]^m \]

where the parameter \( m \) is defined as Ca\textsuperscript{2+} cooperativity. Historically, \( m \), or the Hill coefficient, has been interpreted as the minimal number of Ca\textsuperscript{2+} ions that must cooperatively bind to the Ca\textsuperscript{2+} sensor of the release machinery to trigger a fusion event (Dodge & Rahamimoff, 1967, Hill 1910). Numerous studies, in a variety of synapses, have shown that the \( m \) value usually falls in the 3–4 range, meaning that slight alterations in Ca\textsuperscript{2+} entry into the terminal can profoundly affect synaptic strength as a result of the nonlinearity defined by \( m \). Depending on experimental conditions, the \( m \) value derived from analysis of the input–output relationship of any given synapse provides specific information about the coupling of Ca\textsuperscript{2+} to SV release. For example, the \( m \) value obtained by pairing photolysis uncaging of Ca\textsuperscript{2+} within the terminal itself and measurement of transmitter release most likely reveals the “molecular” cooperativity at the sensor, because this approach bypasses VGCCs and directly targets the binding sites for Ca\textsuperscript{2+} (Bollmann et al., 2000; Schneggenburger & Neher, 2000). If the input–output relationship is based on changes in the number of VGCCs engaged in triggering release, the \( m \) value is then an indirect readout of the spatial interaction between synaptic vesicles (SVs) and Ca\textsuperscript{2+} ions near the inner mouth of open channels (i.e., Ca\textsuperscript{2+} domain) (Borst & Sakmann, 1999; Gentile & Stanley, 2005) and hence may be referred as the “Ca\textsuperscript{2+} channel/domain” cooperativity. Intuitively, one can envision that when VGCCs become spatially closer to a SV, the minimal number of Ca\textsuperscript{2+} domains required to elevate the intracellular Ca\textsuperscript{2+} to the fusion threshold
declines. Therefore, \(\text{Ca}^{2+}\) cooperativity has distinct conceptual meanings in mirroring the coupling nature of \(\text{Ca}^{2+}\) and vesicular release under different experimental circumstances.

To probe developmental plasticity in transmitter release, we have examined the spatial coupling properties of VGCCs and synaptic vesicles at the calyx of Held synapse using brainstem slices taken from postnatal mice before and after the onset of hearing [i.e., postnatal day 11/12 (P11/P12)]. By loading \(\text{Ca}^{2+}\) buffers with distinctive binding kinetics into calyces and by measuring \(\text{Ca}^{2+}\) channel/domain cooperativity with different voltage paradigms, in the absence and presence of subtype-specific toxins for VGCCs, we found that the coupling between VGCCs and synaptic vesicles tightens as maturation progresses. Such changes lead to a switch in release modality from “microdomain,” involving the cooperative action of many loosely-coupled N- and P/Q-type VGCCs, to “nanodomain,” in which opening of fewer tightly-coupled P/Q-type VGCCs is sufficient to induce a fusion event.
3.2 RESULTS

3.2.1 Developmental Difference in Action Potential-Evoked Release of Transmitter.

The quantal output of any given synapse is critically dependent on the shape and size of presynaptic APs, which ultimately determine the amount of Ca\(^{2+}\) influx and transmitter release (Llinás et al., 1982; Augustine, 1990; Sabatini & Regehr, 1997; Borst & Sakmann, 1999).

To examine the effect of AP waveform on Ca\(^{2+}\) currents and transmitter release in developing calyx of Held synapses, we first made current-clamp recordings of APs from immature (P8–P12) and nearly mature (P16–P18) calyces in response to axonal stimulation (Fig. 3.1A). We confirmed that the AP waveform of P8–P12 calyces showed slower rise and repolarization times as well as wider width than that of P16–P18 calyces, as reported previously for the developing calyx of Held synapse in rat (Taschenberger & von Gersdorff, 2000; von Gersdorff & Borst, 2002). Subsequently, we used these waveforms as command templates to perform voltage-clamp recordings of presynaptic Ca\(^{2+}\) currents (I\(_{Ca}\)) (see section 2.2.5) paired simultaneously with recordings of EPSCs (I\(_{EPSC}\)) in 1mM extracellular Ca\(^{2+}\), which is near the physiological concentration of Ca\(^{2+}\) in the brain fluid (Jones & Keep, 1988). This condition also helped improve the quality of the voltage-clamp by reducing the amplitude of Ca\(^{2+}\) currents and I\(_{EPSC}\) and minimized the desensitization of postsynaptic glutamate receptors. When alternating AP waveforms from young (AP\(_i\); amplitude, 110 mV; half width, 0.6 ms) and older (AP\(_M\); amplitude, 110 mV; half width, 0.3 ms) calyces were used as voltage commands in voltage-clamped calyces, we found that Ca\(^{2+}\) currents were effectively evoked by these two protocols in two populations of synapses (Fig. 3.1B,C). The total charge integral of Ca\(^{2+}\) current evoked by either waveform was not different between the two age groups (AP\(_{M}\), 193±24 vs. 230±41 pA•ms; AP\(_i\), 549±18 vs. 555±52 pA•ms for P8–P12 and P16–P18 group, respectively) (Fig. 3.1D). Surprisingly, P8–P12 calyces usually failed to release glutamate in response to AP\(_M\) (no detectable I\(_{EPSC}\) in 13 of 15 calyces) (Fig. 3.1B, right panel), whereas P16–P18 calyces (eight of eight calyces) released consistently with either voltage paradigm, of which AP\(_i\) produced greater I\(_{EPSC}\) than AP\(_M\), as expected (Fig. 3.1C).

These observations raised the possibility that the coupling of VGCCs to vesicular release is fundamentally different in the two age groups of synapses.

3.2.2 Age-Dependent Reduction in EGTA-Induced Attenuation of Transmitter Release.

It has been shown that many VGCCs (in 2mM [Ca\(^{2+}\)]\(_o\)) are required to trigger release of single synaptic vesicles in young calyces (Borst & Sakmann, 1996). In line with this fact, our observation that a substantial Ca\(^{2+}\) influx (in 1mM [Ca\(^{2+}\)]\(_o\)) failed to evoke vesicular release in P8-P12 calyces implies that many of the VGCCs may not be closely-coupled to individual SVs at this developmental stage. However, given the increased release efficacy in P16–P18 synapses, we postulated that presynaptic coupling may tighten during maturation to account for the observed difference between the two groups of synapses.

To test this, we first performed experiments to compare the effectiveness of Ca\(^{2+}\) chelators, EGTA and BAPTA, on reducing quantal output. Despite similar equilibrium dissociation constants, these
Figure 3.1: Profile of Ca$^{2+}$-Dependent Transmitter Release in Response to Action Potentials Recorded From the Developing Calyx of Held.

(A) Action potentials recorded from immature and mature synapses. (B & C) Examples of presynaptic Ca$^{2+}$ currents (Pre-$I_{Ca}$) and EPSCs from postsynaptic neurons (Post-$I_{EPSC}$) simultaneously recorded from P11 (B) and P17 (C) synapses in response to the same set of voltage command waveforms in A. Note that AP$_i$ evoked $I_{Ca}$ but not release from the immature synapse (B, arrow) and that results in B and C were all obtained in 1mM [Ca$^{2+}$]. (D) Pooled Ca$^{2+}$ current areas for both immature and mature test groups in response to both AP$_i$ (open bars) and AP$_m$ (filled bars) waveforms. Abs, Absolute. Error bars represent S.E.M.
two buffers exhibit very different forward-rate constants (~160-fold difference) for binding Ca\(^{2+}\) ions, allowing some inference to be made, based on their differing effectiveness in attenuating synaptic strength, with respect to the distance between VGCCs and vesicles in nerve terminals (Adler et al., 1991; Borst et al., 1995; Naraghi & Neher, 1997; Meinrenken et al., 2002). The rationale of this approach was that if VGCCs were tightly-coupled to vesicles, slow buffers such as EGTA should not be able to intercept Ca\(^{2+}\) ions before they reach the Ca\(^{2+}\) sensor of the release machinery, therefore minimally altering synaptic strength. However, EGTA would effectively attenuate release if VGCCs were physically distant from synaptic vesicles, providing sufficient time for EGTA to bind Ca\(^{2+}\) ions in transit. In contrast, fast buffers such as BAPTA should be able to intercept Ca\(^{2+}\) ions and decrease synaptic strength independent of the tightness of VGCCs and synaptic vesicle coupling.

For this set of experiments, we sealed a patch electrode containing EGTA (10mM) or BAPTA (1mM) onto the presynaptic calyx and collected control \(I_{\text{EPSC}}\) evoked through axonal stimulation (Fig. 3.2Aii). The concentrations of two buffers chosen for these experiments were based on previous experimental results and theoretical simulation (Borst et al., 1995; Borst & Sakmann, 1996; Naraghi & Neher, 1997; Meinrenken et al., 2002). After \(I_{\text{EPSC}}\) baseline measurements, breakthrough of the presynaptic terminal membrane and current-clamp recording mode were sequentially established (Fig. 3.2Aii). After ~2–3 min. allowing diffusion and equilibration of the Ca\(^{2+}\) buffer within the calyx without afferent stimulation, recording of \(I_{\text{EPSC}}\) were then resumed (Fig. 3.2Aiii). When 10mM EGTA was diffused into presynaptic calyces from developing synapses across a P10–P18 age span, we found a strong age dependence in the effectiveness of EGTA on reducing quantal output, being very robust at P10 (~70%) to marginal at P18 (~20%) (mean, 57±4% for P10–P12; 22±4% for P16–P18) (Fig. 3.2B,D). On the contrary, loading of BAPTA (1mM) did not show such a marked age dependence with 43±8 and 43±5% reduction for P10–P12 and P16–P18 calyces, respectively (Fig. 3.2D). The degrees of attenuation caused by these buffers are consistent with those reported previously for young synapses in the rat (P8–P10) (Borst et al., 1995; Borst & Sakmann, 1996). However, our data reveal a strong correlation between the extent of EGTA-induced attenuation and the developmental stage, suggesting that the physical distance between VGCCs and synaptic vesicles indeed tightens with development.

### 3.2.3 Distinctive Ca\(^{2+}\) Cooperativity in P8–P12 & P16–P18 Synapses.

Under our experimental conditions, the relationship between Ca\(^{2+}\) currents and \(I_{\text{EPSC}}\) can be simply described by a power function in the form of \(I_{\text{EPSC}} \propto [I_{\text{Ca}}]^m\) (where \(m\) denotes Ca\(^{2+}\) cooperativity and \(I_{\text{EPSC}}\) as a readout of transmitter release). It is generally accepted that the release of synaptic vesicles often requires the cooperative action of many VGCCs by forming overlapping Ca\(^{2+}\) domains (Luebke et al., 1993; Takahashi & Momiyama, 1993; Wheeler et al., 1994; Wu & Saggau, 1994; Mintz et al., 1995; Iwasaki & Takahashi, 1998). If spatial coupling of VGCCs and synaptic vesicles tightens, as suggested by the EGTA/BAPTA loading experiments, we would predict that the Ca\(^{2+}\) cooperativity decreases as the release of single vesicles becomes less dependent on the cooperative action of VGCCs.
Figure 3.2: Age-Dependent Changes in Spatial Coupling Between VGCCs and Synaptic Vesicles.

(A) Paired recording configurations for Ca\(^{++}\) buffer injections. Postsynaptic whole-cell voltage-clamp recordings coupled with: (i), presynaptic cell-attached mode for baseline measurements; (ii), break-in of presynaptic membrane and buffer infusion in voltage-clamp mode (2–3 min); slight positive pressure was given to ensure thorough dialysis of buffers into the nerve terminal while whole-cell configuration were monitored in voltage-clamp mode; and (iii), presynaptic current-clamp mode at its resting potential (approximately -70mV). EPSCs were evoked through stimulation of the presynaptic axon fiber bundles. Presynaptic APs usually had an overshoot above +10mV without series resistance and capacitance compensation, and their waveform remain unchanged over the recorded time (data not shown). Ca\(^{++}\) chelators (BAPTA or EGTA) were supplemented into the K\(^{+}\)-based intracellular solution and dialysed directly into calyces through the patch pipettes. (B) The amplitudes of EPSCs before and after 10mM EGTA injections were plotted against time for a P12 (top panel) and a P18 (bottom panel) synapse, respectively. Raw traces (insets) with (black line) or without (blue line) EGTA are superimposed for comparison. (C) The extent of EGTA-induced block was plotted against the age of synapses. The block percentage was calculated as the ratio between the mean amplitude of EPSCs after EGTA injections/the mean amplitude of baseline EPSCs (100%) over the duration shown. The solid line represents least-squares linear regression of all data. (D) A summary plot comparing the extent of block induced by EGTA (10mM) and BAPTA (1mM) for P10–P12 and P16–P18 synapses. The asterisk indicates statistical significance (Student’s t-test, p<0.05). Error bars represent S.E.M.
To test this prediction, we compared the effect of AP waveform on Ca\(^{2+}\) currents and quantal output and measured the Ca\(^{2+}\) cooperativity in P8–P12 and P16–P18 synapses. To mimic the changes in AP waveform that takes place in the developing calyx of Held synapse, we gradually increased the width of the AP-like waveform (-70 to +60mV; 100\(\mu\)s increments in repolarization time) (Fig. 3.3A, top panel). It was thought that such changes in spike width would recruit an increasing number of Ca\(^{2+}\) channels and/or prolong their open time without changing the maximal driving force for calcium (Augustine, 1990; Sabatini & Regehr, 1997; Borst & Sakmann, 1999; Pattillo et al., 1999). Figure 3.3A (bottom panel) contrasts two examples of such recordings from P8–P12 and P16–P18 synapses in 1mM [Ca\(^{2+}\)]\(_o\). We observed that the amplitudes of both Ca\(^{2+}\) currents and \(I_{\text{EPSC}}\) increased as the width of AP\(_L\) was increased. We also found that the minimal Ca\(^{2+}\) current at which we could detect quantal events was significantly higher in P8–P12 synapses than that in P16–P18 ones (P8–P12, 470±150 pA; P16–P18, 230±50 pA; \(p<0.008\)) (Fig. 3.3B), suggesting different Ca\(^{2+}\) current thresholds for triggering release in these two populations of synapses. This result is consistent with our observation that P8–P12 terminals failed to release transmitter in response to AP\(_M\) (Fig. 3.1), likely because of the fact that the narrow AP\(_M\) waveform usually generated Ca\(^{2+}\) currents that were sub-threshold for triggering release from P8–P12 terminals but supra-threshold in P16–P18 calyces.

When the normalized area integrals of \(I_{\text{EPSC}}\) were plotted against that of Ca\(^{2+}\) currents on a log–log scale, we determined the Ca\(^{2+}\) cooperativity from the slope of linear fits to these input–output relationships (see section 2.4.2) (Fig. 3.3C). We found that the \(m\)-value for P8–P12 synapses was significantly higher than that for P16–P18 synapses, being 4.8±0.3 and 2.6±0.5, respectively (\(p<0.005\)) (Fig. 3.3C,D). When [Ca\(^{2+}\)]\(_o\) was raised from 1 to 2mM, we observed a decrease in \(m\)-values for both age groups, but a highly significant difference in this parameter was observed in P8–P12 synapses (from 4.8±0.3 to 2.8±0.3; \(p<0.0005\)) (Fig. 3.3D). Our interpretation of these observations is that an increase in the driving force for Ca\(^{2+}\), through individual open channels, reduces the total number of VGCCs or Ca\(^{2+}\) domains required for a fusion event, leading to a decrease in \(m\). Hence, many VGCCs are likely required to trigger release of single vesicles in young synapses, whereas tighter coupling in older synapses reduces the number of VGCCs or Ca\(^{2+}\) domains required for a release event.

Because the pseudo-AP\(_L\) waveforms used above had different repolarization phases, it is conceivable that not only the number of VGCCs being recruited but other confounding factors may also contribute to the different efficacy of vesicular release. For example, the density and voltage dependence of these channels in P8–P12 and P16–P18 calyces may also differ. To address these issues, we next examined the properties of VGCCs with a series of command voltage-steps (10ms steps from -70 to +60mV in 10mV increments). We found that the two sets of current-voltage relationships were virtually identical, in that Ca\(^{2+}\) currents from both age groups activated at approximately -40 mV and reversed around +40 mV with an identical maximal current at -10 mV (Fig. 3.4A,B). These results suggest that both the total number and voltage dependence of VGCCs are very similar for both age groups of calyces. In line with this, the maximal tail currents evoked during this protocol were not statistically different in amplitude (1.5±0.1nA for P8–P12; 1.2±0.2nA for P16–P18; \(p>0.1\)) and occurred at approximately +20mV for both age groups.
Figure 3.3: Measurement of Ca\(^{2+}\) Cooperativity With Action Potential-Like Waveforms.

(A) Examples contrasting two paired recordings of presynaptic Ca\(^{2+}\) currents (Pre-\(I_{\text{Ca}}\)) and EPSCs from postsynaptic neurons (Post-\(I_{\text{EPSC}}\)) from P9 (middle panel) and P18 synapses (bottom panel) in response to pseudo-action potential-like voltage ramps (-70 to +60mV; rise time, 0.2ms; falling phase, from 0.2 to 0.6ms with 0.1ms increments). (B) The mean amplitude of threshold Pre-\(I_{\text{Ca}}\) at which quantal release events were detected in P8–P12 and P16–P18 synapses. (C) Area integral of Pre-\(I_{\text{Ca}}\) and I\(_{\text{EPSC}}\) from two recordings in A are normalized (norm) and plotted on a log–log scale. Solid lines are linear regression of paired data for two synapses and \(m\)-values given. Note that results from A–C were all obtained in 1mM [Ca\(^{2+}\)]. (D) A summary plot of cooperativity \(m\)-values in 1 and 2mM [Ca\(^{2+}\)] from two populations of synapses. Single or double asterisks in B and D indicate statistical significance (Student’s t-test, \(p<0.05\)) between P8–P12 and P16–P18 groups or within the same age group, respectively. Error bars represent S.E.M.
Figure 3.4: Voltage Dependence of VGCCs in P8–P12 & P16–P18 Calyces.  
(A) Example recordings of presynaptic Ca\(^{2+}\) currents (Pre-I\(_{\text{ca}}\)) in response to voltage steps (10ms) from -70 to +60mV in 10mV increments (top panel) from a P11 (middle panel) and a P17 (bottom panel) synapse in 1mM [Ca\(^{2+}\)].  
(B) Current–voltage relationships of Pre-I\(_{\text{ca}}\) from P8–P12 (open diamonds) and P16–P18 calyces (filled diamonds). Error bars represent S.E.M.
Knowing that VGCCs in both groups of terminals have similar properties, we used another voltage-clamp protocol \((\text{AP}_D; -70 \text{ to } +60 \text{mV}; 100 \mu\text{s increments in plateau time})\) (Fig. 3.4A, top) to further explore the presynaptic coupling between VGCCs and vesicular release. Incremental increases in plateau duration were used to recruit an increasing number of VGCCs while maintaining the driving force for \(\text{Ca}^{2+}\) as well as the on and off kinetics of the command voltage steps. Because voltage steps to +60mV in \(\text{AP}_D\) presumably maximized the open probability of VGCCs, while the driving force for \(\text{Ca}^{2+}\) remains constant for all open channels (Augustine et al., 1987; Roberts, 1994; Borst & Sakmann, 1998; Borst & Sakmann, 1999; Gentile & Stanley, 2004; King & Meriney, 2005), the only variable at any given plateau duration would be the number of VGCCs engaged in mediating release of synaptic vesicles. Using these \(\text{AP}_D\) waveforms, we compared the relationships between \(I_{\text{Ca}}\) and \(I_{\text{EPSC}}\) and determined \(m\)-values for P8–P12 and P16–P18 synapses in 1 and 2mM \([\text{Ca}^{2+}]_0\) (Fig. 3.5A,B).

We again found that the \(m\)-values from P8–P12 synapses were significantly higher than those of P16–P18 ones \((5.5\pm0.5 \text{ for P8–P12, } 3.0\pm0.4 \text{ for P16–P18 synapses in } 1\text{mM } [\text{Ca}^{2+}]_0; p<0.0004)\) and that raising \([\text{Ca}^{2+}]_0\) from 1 to 2mM led to a reduction in \(m\)-values for both populations of synapses, producing values consistent with that reported previously under similar conditions (Borst and Sakmann, 1999) \((3.5\pm0.5 \text{ for P8–P12, } 2.1\pm0.2 \text{ for P16–P18 synapses in } 2\text{mM } [\text{Ca}^{2+}]_0; p<0.02)\) (Fig. 3.5C). Moreover, we found no difference in the rise or decay time of \(\text{Ca}^{2+}\) currents evoked by the \(\text{AP}_D\) protocol between two populations of calyces \((10–90\% \text{ rise time, } 0.18\pm0.02 \text{ vs. } 0.15\pm0.01 \text{ms, } p>0.2; 10–90\% \text{ decay time, } 0.51\pm0.05 \text{ vs. } 0.56\pm0.04 \text{ms, } p>0.5)\) (Fig. 3.5D), indicating that the difference in \(m\) is independent of gating kinetics of VGCCs.

The calyx of Held synapse undergoes dramatic morphological refinement over the first two postnatal weeks (Morest, 1968; von Gersdorff & Borst, 2002). The “spoon-shaped” calyx at early postnatal stages may slow glutamate removal, whereas older “finger-digit-like” terminals allow more rapid clearance of glutamate from the synaptic cleft. Because we use AMPA receptor (AMPA) \(I_{\text{EPSC}}\) as a readout of transmitter release, different residence times of transmitter in P8–P12 and P16–P18 synapses may lead to different extents of AMPAR desensitization (Joshi and Wang, 2002; Taschenberger et al., 2002), potentially contributing to the difference in the \(m\)-value between the two age groups. To test this, we repeated the experiments in 1mM \([\text{Ca}^{2+}]_0\) with the \(\text{AP}_D\) paradigm in the presence of cyclothiazide (CTZ; 50\mu M), which blocks AMPAR desensitization. Despite application of CTZ, the age-dependent difference in \(m\) remained \((5.5\pm0.7 \text{ for P8–P12, } 2.9\pm0.1 \text{ for P16–P18 synapses; } p<0.01)\) (Fig. 3.5C), indicating that the developmental difference in \(\text{Ca}^{2+}\) cooperativity is independent of postsynaptic receptor desensitization.

Because temperature may profoundly affect synaptic efficacy at this synapse (Taschenberger & von Gersdorff, 2000; von Gersdorff & Borst, 2002), we next measured the \(\text{Ca}^{2+}\) cooperativity in 1mM \([\text{Ca}^{2+}]_0\) at near-physiological temperature \((35^\circ\text{C})\). We found that an increase in temperature reduced the \(m\)-value significantly in young synapses but only slightly in older ones (Fig. 3.5C). However, a significant difference in the \(m\)-value between the two age groups remained \((3.3\pm0.1 \text{ for P8–P12, } 2.5\pm0.2 \text{ for P16–P18 synapses in } 1\text{mM } [\text{Ca}^{2+}]_0; p<0.002)\). These results led us to suggest that raising temperature may
Figure 3.5: Comparison of Ca²⁺ Cooperativity Independent of Gating Kinetics of VGCCs & Ca²⁺ Driving Force.

(A) Example recordings of presynaptic Ca²⁺ currents (Pre-\( I_{Ca} \)) in response to voltage steps with increasing plateau duration (top; -70 to +60mV; rise and fall time, 0.2ms; plateau duration, from 0 to 0.5ms in 0.1ms increments) and \( I_{EPSC} \) from a P10 (middle panel) and a P17 (bottom panel) synapse in 1mM [Ca²⁺].

(B) Area integral of Pre-\( I_{Ca} \) and \( I_{EPSC} \) from recordings in A are normalized (norm) and plotted on a log–log scale. Solid lines are linear regressions of paired data for two synapses and \( m \)-values given.

(C) A summary plot of cooperativity (\( m \)) values from two age groups under four given experimental conditions (insets). Single or double asterisks in C indicate statistical significance (Student’s t-test, \( p<0.05 \)) between the P8–P12 and P16–P18 groups or within the same age group.

(D) The 10–90% rise and decay time of the maximal Pre-\( I_{Ca} \) evoked by AP are summarized for two age groups of synapses. Error bars represent S.E.M.
lead to an expansion of individual Ca\textsuperscript{2+} domains, presumably as a result of increased channel conductance and Ca\textsuperscript{2+} diffusion rate. Such a manipulation reduces the number of Ca\textsuperscript{2+} channels required to trigger single fusion events in P8–P12 synapses, similar to the effect of raising [Ca\textsuperscript{2+}]\textsubscript{o}. In contrast, temperature has little effect on P16–P18 synapses, because the tight coupling of VGCCs and synaptic vesicles minimizes the dependence on cooperative action of multiple Ca\textsuperscript{2+} domains, independent of increases in Ca\textsuperscript{2+} domain size and diffusion rate. Collectively, the results acquired with the AP\textsubscript{D} paradigm reinforce our interpretation of age-dependent decreases in the Ca\textsuperscript{2+} channel/domain cooperativity, based on the AP\textsubscript{L} protocol. As such, the number of VGCCs or Ca\textsuperscript{2+} domains required for release of single synaptic vesicles decreases as the calyx of Held synapse matures.

### 3.2.4 The Role of Ca\textsuperscript{2+} Channel Types in Developmental Transformation of the Release Modality.

Previous studies have shown that multiple types of VGCCs (N, P/Q, and R-type) are present in young calyces and cooperatively mediate transmitter release (Wu et al., 1998; Wu et al., 1999); however, only P/Q-type VGCCs exclusively mediate transmitter release in older ones (Iwasaki & Takahashi, 1998). Immunofluorescence staining of young calyces with subtype-specific antibodies further revealed that N- and R-type VGCCs appear to be physically more distant from release sites than P/Q-type (Wu et al., 1999), raising the possibility that subtypes of VGCCs may be differentially involved in the developmental reorganization of channels and synaptic vesicles.

To test this possibility, we measured Ca\textsuperscript{2+} cooperativity in the presence of toxins that specifically block different types of Ca\textsuperscript{2+} channels in young synapses (P8–P12). This age window covered the critical period of developmental transition in VGCCs engaged in triggering vesicular fusion, being from N-, R-, and P/Q-type channels together to P/Q-type alone after P10. By dividing these immature synapses into two age subgroups (P8/9 vs. P11/12), we found that blocking P/Q-type Ca\textsuperscript{2+} channels with ω-agatoxin-IVA (100nM) did not change the m-value (5.6±0.4 for P8–P12 in 1mM [Ca\textsuperscript{2+}]\textsubscript{o}), despite a sharp reduction in quantal output (Fig. 3.6). However, when N-type Ca\textsuperscript{2+} channels are blocked with ω-conotoxin-GVIA (1μM), we found a significant difference in m-value within two subpopulations of young synapses, the P11/12 subgroup being significantly lower (3.8±0.2; p<0.03) in comparison with that of the control (no toxin) group (5.5±0.5 for P8–P12), whereas the m-value for P8/9 subgroup was unchanged (5.3±0.5) (Fig. 3.6B,C).

These observations suggest that N-, P/Q-, and probably R-type Ca\textsuperscript{2+} channels are spatially intermingled in the active zone (AZ) at P8/9 and that release of single vesicles relies on the cooperative action of many channels, regardless of Ca\textsuperscript{2+} channel subtype. As maturation progresses, P/Q-type VGCCs predominate over N- and R-type VGCCs (Wu et al., 1999) and become more tightly-associated with vesicles, capable of triggering release with a lower m-value, even as early as P11/12, when N-type channels are blocked. It should be noted that previous studies have simultaneously measured changes in the volume averaged Ca\textsuperscript{2+} concentration and transmitter release during application of a toxin that gave an estimate of the effectiveness (also termed as m or n) for the toxin-sensitive subtype of VGCCs to induce quantal release (Wu and Saggau, 1994; Mintz et al., 1995; Wu et al., 1999). In general, these
Figure 3.6: The Role of Subtypes of VGCCs in Regulating Ca$^{2+}$ Cooperativity in P8–P12 Synapses.

(A) Example recordings of presynaptic Ca$^{2+}$ currents (Pre-I$_{\text{Ca}}$) in response to voltage steps with increasing plateau duration (top; -70 to +60mV; rise and fall time, 0.2ms; plateau duration, from 0 to 0.5ms in 0.1ms increments) and I$_{\text{EPSC}}$ from a P10 in $\omega$-agatoxin-IVA (w/$\omega$-atx; 100nM) (top panel) and a P8 synapse $\omega$-conotoxin-GVIA (w/$\omega$-ctx; 1µM) (bottom panel). (B) A log–log plot of normalized area integral of Pre-I$_{\text{Ca}}$ and I$_{\text{EPSC}}$ from four recordings including those two in A. Solid lines are linear regressions of paired data for each recording and m-values given. (C) A summary plot of cooperativity m-values in control, $\omega$-agatoxin-IVA group, and two subgroups (P8/9 vs P11/12) in $\omega$-conotoxin-GVIA. The single asterisk indicates statistical significance (Student’s t-test, p<0.05) between two subgroups of young synapses. All experiments were performed in 1mM [Ca$^{2+}$]. Note low yield of transmitter release after addition of either toxin. Error bars represent S.E.M.
values of $m$ or $n$ are high (3-4) when P/Q-type VGCCs are gradually blocked with $\omega$-agatoxin-IVA and are low (1–2) when other subtypes are blocked in a similar manner. Such experiments reveal the nature of the cooperation between different subtypes of VGCCs in triggering release. Our $m$-values, derived by AP$_D$ paradigm, after a complete blockade of one subtype of VGCCs by a toxin, has a distinct meaning in that the cooperative domain interactions among the remaining type(s) of VGCCs are assayed.

3.2.5 Mixed Populations of Loosely- & Tightly-Coupled Synaptic Vesicles in Developing Calyces.

Our observations that EGTA (10mM) did not completely attenuate transmitter release at any given age and that the extent of this attenuation was strongly correlated with synaptic maturity led us to postulate that in developing synapses, there are at least two populations of release-competent synaptic vesicles, one loosely-coupled and the other tightly-coupled to VGCCs. The relative proportion of the latter may be increasing with development, leading to a decrease in $m$. If the loosely-coupled population of channel-vesicle units were functionally removed from young terminals, one would predict that the remaining population of tightly-coupled vesicles be released with a similar $m$ to that of older synapses. To test this prediction, we measured $m$ from P8–P12 synapses after disabling loosely-coupled vesicles through presynaptic loading of 10mM EGTA (Fig. 3.7A,B). We found that quantal output was low under such conditions, but the $m$-value (3.3±0.3; 10mM EGTA) decreased to that typical of P16–P18 synapses (3.0±0.4; 0.5mM EGTA) (Fig. 3.7B,C, 3.5C), in which tightly-coupled vesicles presumably dominate (~80%) as inferred from the reduction induced by 10mM EGTA (Fig. 3.2C,D). This experiment appears to have similar end effects to that with application of $\omega$-conotoxin-GVIA, suggesting, under both manipulations, release of transmitter becomes primarily dependent on tightly-coupled, low-$m$ units predominately containing VGCCs of the P/Q-type. Hence, the release modality of young synapses, after loading of 10mM EGTA or $\omega$-conotoxin-GVIA (older than P11/12), can be functionally converted to that reminiscent of older synapses.
Figure 3.7: Transformation of Release Modality From Microdomain to Nanodomain Coupling.

(A) An example recording of presynaptic Ca\(^{2+}\) currents (Pre-I\(_{\text{Ca}}\)) in response to AP\(_D\) paradigms (top; -70 to +60mV; rise and fall time, 0.2ms; plateau duration, from 0 to 0.5ms in 0.1ms increments) and I\(_{\text{EPSC}}\) from a P9 synapse in 1mM [Ca\(^{2+}\)]. Intracellular solution for presynaptic recordings contains 10mM EGTA. 

(B) Area integral of Pre-I\(_{\text{Ca}}\) and I\(_{\text{EPSC}}\) from recordings in A are normalized (norm) and plotted on a log–log scale. The solid line represents the linear fit to the paired data set and \(m\)-value given. Dashed line from the same experiment as in Figure 3.5B with low concentration of intracellular EGTA (0.5mM) was plotted for comparison. 

(C) A summary plot of cooperativity \(m\)-values in 1mM [Ca\(^{2+}\)], with 0.5 or 10mM EGTA in presynaptic calyces from the P8–P12 synapses. The asterisk indicates statistical significance (Student’s t-test, \(p<0.05\)) between two data sets. As a result of the low quantal output with high variance after 10mM EGTA infusion into some of P8–P12 synapses, multiple experimental runs were taken and overlaid to derive a single \(m\)-value. Error bars represent S.E.M.
3.3 DISCUSSION

The classical definition of Ca\(^{2+}\) cooperativity usually refers to the cooperative action of Ca\(^{2+}\) ions at the Ca\(^{2+}\) sensor of the release machinery (e.g., synaptotagmin), to which a minimum of three or four Ca\(^{2+}\) ions must bind before fusion takes place (Dodge and Rahamimoff, 1967).

In this study, we measured Ca\(^{2+}\) cooperativity in P8–P12 and P16–P18 synapses using voltage paradigms, AP\(_D\) in particular, to specifically recruit different numbers of VGCCs and generate graded Ca\(^{2+}\) currents and quantal output. Our results imply that the \(m\)-value is an index of the contribution of multiple Ca\(^{2+}\) channels or domains (i.e., Ca\(^{2+}\) channel or domain cooperativity) under this experimental condition.

It is reasonable to extrapolate from channel recruitment experiments that a number of Ca\(^{2+}\) domains are needed to increase the likelihood of three or four Ca\(^{2+}\) ions binding to the sensor, when individual Ca\(^{2+}\) domains are small (as in 1mM \([\text{Ca}\(^{2+}\)]_o\)) and distant from the vesicle (as in young calyces). Because the spatial distance between channel and vesicle shortens during maturation, the number of domains required for three or four Ca\(^{2+}\) ions to reach the sensor declines. The fact that an increase in domain size, by raising \([\text{Ca}\(^{2+}\)]_o\) or by facilitating Ca\(^{2+}\) diffusion through increases in recording temperature (Fig. 3.5), leads to a decrease in the \(m\)-value further supports the interpretation that Ca\(^{2+}\) cooperativity in this study is likely related to the number of Ca\(^{2+}\) channels or domains. Our results demonstrate that a majority of vesicles in young terminals are loosely-coupled to VGCCs, showing high cooperativity values [i.e., \(m=4.8\) (AP\(_L\)) or 5.5 (AP\(_D\)) in 1mM \([\text{Ca}\(^{2+}\)]_o\)] and a high sensitivity to EGTA.

As in several other synapses (Luebke et al., 1993; Takahashi & Momiyama, 1993; Wheeler et al., 1994; Wu & Saggau, 1994; Mintz et al., 1995), the release of single vesicles at the young calyx of Held synapse depends on the cooperative action of overlapping Ca\(^{2+}\) domains from many channels (Borst & Sakmann, 1996, Borst & Sakmann, 1999; Meinrenken et al., 2002) and likely different subtypes of VGCCs such as N, R, and P/Q-types (Iwasaki & Takahashi, 1998; Wu et al., 1999). R-type VGCCs mainly localized on the uninnervated side of the calyx, and therefore it is unlikely that they contribute significantly to phasic release when the other two types are present (Wu et al., 1999). Our Ca\(^{2+}\) channel/domain cooperativity values are consistent with those from previous studies performed in the rat calyx of Held synapses at the same developmental stage (P8–P10) under similar experimental conditions (i.e., \(m\)-values of \(~3–4\) in 2mM \([\text{Ca}\(^{2+}\)]_o\)) (Borst & Sakmann, 1999). However, our \(m\)-values in 1mM \([\text{Ca}\(^{2+}\)]_o\) (4.8–5.5) are higher than those obtained by photolysis uncaging of Ca\(^{2+}\) (4.2–4.4) (Bollmann et al., 2000; Schneggenburger & Neher, 2000), which may represent a theoretical ceiling for molecular cooperativity (Gentile & Stanley, 2004), because the latter measurements are independent of \([\text{Ca}\(^{2+}\)]_o\) and the number of VGCCs. Perhaps different spatiotemporal profiles of Ca\(^{2+}\) ions released from the cage and influx through VGCCs underlies this discrepancy. Alternatively, this theoretical ceiling may be an underestimate of true molecular cooperativity as more than one SNARE complex is thought to be required for fusion of a SV (Weber et al., 1998; Montecucco et al., 2005; Jahn & Scheller, 2006). In fact, at least five binding sites for Ca\(^{2+}\) ions on the sensor were required to fit the data in modeling based on photolysis uncaging experiments (Bollmann et al., 2000; Schneggenburger & Neher, 2000).
Surprisingly, we found that as the synapse matures, the physical distance between VGCCs and single vesicles appears to shorten, as inferred by a decline in the effectiveness of EGTA on attenuating release. The low $m$-values found in older synapses (P16–P18; $m=2.1–2.6$ in 2mM $[\text{Ca}^{2+}]_o$) and the marked reduction in sensitivity to EGTA further suggest that the release modality between VGCCs and single vesicles shifts from primarily overlapping microdomain to nanodomain in which very few channels are required for release of a single vesicle (Fig. 3.8A,B), similar to those from the squid giant synapse, frog neuromuscular junction, and the chick calyx in the ciliary ganglion (Llinás et al., 1981; Llinás et al., 1982; Yoshikami et al., 1989; Augustine, 1990; Stanley, 1993; Wachman et al., 2004; Gentile & Stanley, 2005). However, we would emphasize that use of the terms microdomain and nanodomain in our study specifically refers to differences in the spatial distance between the source of $\text{Ca}^{2+}$ entry and a SV but does not differentiate whether a nanodomain contains one $\text{Ca}^{2+}$ channel or more than one $\text{Ca}^{2+}$ channel.

It is conceivable that $\text{Ca}^{2+}$ domains from the simultaneous opening of two neighboring channels, in a cluster of VGCCs, may pool to form a single domain. Unlike other synapses that contain highly organized arrays of VGCCs and SVs, the distribution pattern of VGCCs on the release face of the calyceal terminal is not known at the calyx of Held synapse. Modeling studies suggest that VGCCs, when placed as a cluster or scattered, may produce entirely different profiles of $\text{Ca}^{2+}$ transients and transmitter release (Roberts, 1994; Meinrenken et al., 2002). It is possible that the spatial distribution of VGCCs transforms from scattered in immature synapses to highly organized arrays in mature ones. As a consequence of such a developmental transformation, the relative portion of tightly-coupled versus loosely-coupled channel vesicle units increases (Fig. 3.8), so that the slow buffer EGTA becomes less effective in attenuating transmitter release as synapses mature.

Our results do not exclude the possibility that other developmental changes, such as the expression level and makeup of $\text{Ca}^{2+}$ sensor proteins for release (e.g., synaptotagmin), may increase the sensitivity of the release machinery to $\text{Ca}^{2+}$ and also contribute to differences in release efficacy (Stewart et al., 2000; Sugita et al., 2001). Transmitter release in young calyces is already highly sensitive to intracellular $\text{Ca}^{2+}$ (~10μM) (Bollmann et al., 2000; Schneggenburger & Neher, 2000). However, it is unknown whether there is a concurrent change in the sensitivity of the release machinery in the developing calyx of Held synapse. Direct measurement of the $\text{Ca}^{2+}$ sensitivity in the future, through photolysis of caged $\text{Ca}^{2+}$ compounds independent of VGCCs, may provide compelling answers to this question. It should also be noted that the contribution of mobile endogenous $\text{Ca}^{2+}$ buffers, with potentially different developmental profiles, likely contributes little to the interpretation of these data due to the rapid dialysis of the exogenous buffers contained within the pipette into both immature and mature terminals.

The evidence in this study, and that from the literature, favors our view that developmental changes in spatial coupling is likely one of the main elements in determining the difference in $\text{Ca}^{2+}$ cooperativity between young and older synapses. The strong correlation between EGTA-induced attenuation of transmitter release and maturity of synapses suggests a spatial/physical tightening of channels and vesicles. Pharmacological transformation of young synapses to older ones, in terms of $\text{Ca}^{2+}$ cooperativity, through presynaptic loading of 10mM EGTA (Fig. 3.7), reinforces this idea.
Figure 3.8: Model Diagrams Illustrating Developmental Differences in the Presynaptic Arrangement of VGCCs and Synaptic Vesicles in Young & Older Synapses.  
(A) Most vesicles in young (left panel) synapses are loosely coupled to VGCCs to form larger functional units (Loosely Coupled Units, LCU) than that of older synapses (right panel), in which a majority of vesicles are tightly coupled to VGCCs (Tightly Coupled Units, TCU). (B) Microdomain (left panel) and nanodomain (right panel) release modalities in young and older synapses. The former requires cooperative actions of multiple Ca\(^{2+}\) channels to trigger release of a single vesicle, whereas the latter implies that VGCCs in older synapses are physically close to synaptic vesicles, and activation of few VGCCs enables release of a single vesicle.
Experiments with subtype-specific toxins suggest that P/Q-type channels appear to drive the development of the nanodomain modality (Fig. 3.6), implying developmental changes in Ca\textsuperscript{2+} cooperativity is likely a "channel phenomenon". This is also in line with the notion that VGCCs physically interact with synaptic proteins (i.e., soluble N-ethylmaleimide-sensitive factor attachment protein receptors) in the active zone of nerve terminals, and these two elements can reciprocally regulate each other once in a complex (Augustine et al., 2003). Emerging evidence from other synapses demonstrates that developmental evolution of release modality involves a switch in the subtype of VGCCs, being from N-type in young synapses to predominantly P/Q-type in older synapses (Reid et al., 2003). In fact, N-type VGCCs fail to fully compensate for synaptic deficits, in P/Q-channel knock-out mice, in central synapses including the calyx of Held synapse (Jun et al., 1999; Inchauspe et al., 2004). This is in line with the view that the P/Q-type Ca\textsuperscript{2+} channels may occupy preferred "slots" for mediating vesicular fusion in the active zone (Cao et al., 2004).

In addition to the release modality transformation, it is also important to note that the morphological structure of the calyx of Held undergoes a significant transformation from spoon shape to finger digit-like (Morest, 1968; von Gersdorff & Borst, 2002). By P14, the morphological structure of calyces is considered to be fully mature. In the rat calyx of Held, the number of active zones doubles (from ~300 to ~600), whereas the size of each AZ decreases, during early postnatal stages (less than P14), as shown by analysis of presynaptic structures using electron microscopy (Taschenberger et al., 2002). However, without precise knowledge of the number of AZs, single-channel conductance of P/Q-type channels at physiological concentrations of [Ca\textsuperscript{2+}]\textsubscript{o} and the spatial distribution patterns of VGCCs, it is perhaps premature to assign a specific number of channels to each AZ. However, recent evidence suggests that ~35% and ~50% of available VGCCs are activated by AP\textsubscript{M} and AP\textsubscript{I} waveforms respectively (Yang & Wang, 2006). This implies that a lower limit of three VGCCs must be in each AZ of the mature synapse in order to have the potential of measuring an average ~30% activation of VGCCs per-AP.

Assuming a similar increase in the number of AZs at the mouse calyx of Held synapse during development (~double), it is conceivable that each AZ contains fewer VGCCs in later developmental stages, given that the maximal Ca\textsuperscript{2+} current appears to be the same between the two age groups. Thus, the lower limit for the number of VGCCs in the immature AZ would be approximately six. In parallel with dramatic changes in morphology after the onset of sensory input (P11/12), similar total numbers of VGCCs spread among an increasing number of AZs may underlie the transformation to a nanodomain release modality in older synapses.

Because the relationship between Ca\textsuperscript{2+} influx and quantal output (I\textsubscript{EPSC}) is highly nonlinear at this synapse ($I_{EPSC} \propto [I_{Ca}]^m$), small changes in either $I_{Ca}$, $m$, or both, may have a profound effect on quantal output. At the calyx of Held synapse, developmental shortening of AP width would reduce the amount of Ca\textsuperscript{2+} influx, whereas transformation of the release modality from microdomain to nanodomain decreases the $m$-value. These two effects would ultimately converge to reduce the release probability of the synapse, as has been reported previously (Iwasaki & Takahashi, 2001; Taschenberger et al., 2002).
The calyx of Held synapse, at maturity, is capable of phase-locking postsynaptic firing to presynaptic input at high rates, as a result of multiple adaptations (Wang & Kaczmarek, 1998; Trussell 1999; Joshi & Wang, 2002; von Gersdorff & Borst, 2002; Joshi et al., 2004). In line with previous evidence showing a developmental increase in the efficiency of exocytosis (Taschenberger et al., 2002), we suggest that tight coupling between VGCCs and vesicles may represent a critical adaptation that ensures a strong synaptic drive for high-fidelity neurotransmission with minimal jitter in synaptic delay. The low-$m$ value associated with nanodomain release modality may also constrain the effect of residual $\text{Ca}^{2+}$ build-up on quantal output during repetitive synaptic activity and thus prevent depletion of synaptic vesicles. Hence, tightening the presynaptic coupling between VGCCs and single synaptic vesicles may have a profound impact on synaptic strength and fidelity during the critical period of development in this and other central synapses.
4. ACTIVITY-DEPENDENT CHANGES IN TEMPORAL COMPONENTS OF NEUROTRANSMISSION AT THE JUVENILE CALYX OF HELD SYNAPSE
4.1 INTRODUCTION

Since the early studies of Bernard Katz et al. in the 1960s (Katz, 1969), a large body of work has established that the process of fast neurotransmission, across a variety of chemical synapses, is highly conserved. Action potentials (APs) in the presynaptic cell are usually initiated from the axon hillock and propagate along the axon. Upon arrival of an AP at the nerve terminal, voltage-gated Ca\(^{2+}\) channels (VGCCs) open and flux Ca\(^{2+}\) ions, raising intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{Bi}}\)) and triggering engagement of release machinery (i.e. SNAREs) on synaptic vesicles (SVs) and within active zones (AZs). This process leads to membrane fusion and ultimately unloading of neurotransmitter from SVs. When transmitters diffuse across the synaptic cleft, they bind to, and activate, ligand-gated ion channels in the postsynaptic density, generating responses in the target neuron. Over the last several decades, tremendous insight has been gained into the mechanisms underlying the regulation of synaptic strength and plasticity by Ca\(^{2+}\) influx (Augustine et al., 1987; Zucker, 1996; Meinrenken et al., 2003; Schneggenburger & Neher, 2005). Despite these advances, little is known about how or even whether, the temporal delays associated with the various steps in neurotransmission exhibit plasticity (Lin & Faber, 2002). Lack of understanding in this regard is particularly evident for mammalian central synapses which are small in size, difficult to access with electrodes, and have thus largely precluded quantitative delineation of the various temporal components of neurotransmission. Given that the fidelity of neurotransmission at any synapse depends both on the magnitude and timing of synaptic inputs, understanding the temporal building blocks for signal propagation and transduction is therefore of central importance in understanding the physiology of synapses.

Classical studies at the frog neuromuscular junction (NMJ) indicated that the latency distribution histogram of synaptic delay, which refers to the time interval between the arrival of an AP at the nerve terminal and the onset of a postsynaptic response, is stable under a variety of conditions (Katz & Miledi, 1965; Barrett & Stevens, 1972; Datyner & Gage, 1980; Parnas et al., 1989). However, recent evidence from other synapses, including the goldfish Mauthner cell synapse and crayfish NMJ, suggests that synaptic delay (SD) is modifiable. Paired-pulse depression in the Mauthner cell synapse is associated with a prolongation in SD (Waldeck et al., 2000), while facilitation at the crayfish NMJ leads to an activity-dependent shortening in SD (Vysheskiyi et al., 2000). These studies raise the possibility that the temporal components of neurotransmitter release, including SD, may undergo changes, either in parallel with or independently of, changes in synaptic strength.

The calyx of Held synapse is an axosomatic synapse located in the medial nucleus of the trapezoid body (MNTB) and specialized for preserving timing information along sound localization pathways in the auditory brainstem (Trussell, 1999; von Gersdorff & Borst, 2002). This synapse is known to be capable of high-fidelity transmission at extraordinarily high rates (up to several hundred Hz). The giant size of this synapse permits direct simultaneous recordings of both pre- and postsynaptic responses, presenting an excellent model to clearly dissect various temporal delays in the context of its physiological functionality (Forsythe, 1994; Borst et al., 1995). Using paired recordings from the mouse calyx of Held-MNTB synapse in acute brainstem slices, we show here that stimulation of the presynaptic axon, at a broad spectrum of frequencies, leads to activity-dependent prolongation in certain temporal
components of neurotransmission. Build-up of intra-terminal Ca\(^{2+}\), and depletion of SVs in the readily-releasable pool during repetitive stimulation, likely underlie this activity-dependent short-term temporal plasticity.
4.2 RESULTS

4.2.1 Definition of the Temporal Components of Synaptic Transmission.

To experimentally separate the timing landmarks of signal propagation at the calyx of Held-MNTB synapse, we made dual-electrode recordings in which one electrode was sealed onto the presynaptic membrane, in the cell-attached voltage-clamp configuration, while the other electrode was used to establish a whole-cell voltage-clamp configuration in the postsynaptic neuron. We chose to use the cell-attached configuration, rather than whole-cell current-clamp recordings of presynaptic APs, to minimize disruption of intra-terminal homeostasis while maintaining long-lasting recordings. Presynaptic axons were stimulated with a platinum bipolar electrode placed less than 200 µm away from the recorded cells. Figure 4.1A shows an example of simultaneous pre- and postsynaptic recordings in response to a single stimulus. Following the stimulation artifact, the presynaptic pipette registered an AP invasion in the form of a compound current with inward and outward deflections, reflecting the depolarization and repolarization phases of the AP. The peaks of these currents, corresponding to the time point at which the maximal rate of AP depolarization/repolarization occurs, approximate the AP half-width (Sabatini & Regehr; 1997; Yang & Wang, 2006). Shortly afterwards, an excitatory postsynaptic current (I EPSC) was recorded from the postsynaptic neuron. From such an experimental arrangement, we separated three temporal components of transmission delay (TD), as illustrated in Fig. 4.1A. (1) Conduction Delay (CD): the time interval between the peak of the stimulation artifact and that of the presynaptic inward current deflection, reflecting the time required for AP propagation; (2) Synaptic Delay (SD): the time interval between the peak of the presynaptic inward current deflection and the onset of an EPSC in the postsynaptic cell, representing the time required for Ca 2+-dependent vesicular release of transmitter and the diffusion of that transmitter to postsynaptic receptors; and (3) Response-Rise Delay (RRD): the time interval between the onset of I EPSC and its peak, providing a measure of the synchronicity of release events.

Amongst the three delay components, accurate determination of SD is the most difficult to achieve. The onset of I EPSC, unlike other measurement points with defined peaks, has no clear inflection as a reference. Two previous methods have been used to determine the onset of single I EPSC (Fig. 4.1B). The first method is based on setting an arbitrary proportion of the I EPSC peak (e.g. 5 or 10%), at which the time point for the I EPSC onset is taken (Taschenberger & von Gersdorff, 2000) (Fig. 4.1B, top panel, named herein as the “Threshold Method”). The second takes the intersection point of two linear regression lines, one fit to the baseline and the other fit to the rise phase of the I EPSC (10-90%), as the onset of I EPSC (Wu & Borst, 1999) (Fig. 4.1B, bottom panel, named herein as the “Intersection Method”). However, both methods tend to carry significant errors and methodological bias, especially when the I EPSC rising-phase is shallow, which makes their accuracy questionable under certain circumstances (see section 2.5 and Fig. 2.1). Here, we developed a novel method for determining the onset of I EPSC, which improves the degree of detection accuracy and eliminates rise phase-dependent bias. In this method, we define the onset of an I EPSC as the point of maximal curvature along the I EPSC rise phase. To mathematically resolve the maximal curvature point, we first fit the rise phase of the I EPSC with an equation of the form of the Boltzmann charge-voltage function (Fig. 4.1C, left panel) and then solve this
Figure 4.1: Experimental Arrangement & Analytical Approaches For Quantifying Temporal Delays of Synaptic Transmission.

(A) Diagram illustrating simultaneous recording from a presynaptic calyx, in cell-attached configuration, and a postsynaptic neuron in whole-cell configuration (top panel). Example current traces (middle panel) are also shown, on which the deflections are marked with dotted lines to define conduction delay (1/CD), synaptic delay (2/SD), response rise delay (3/RRD), and transmission delay (4/TD). (B) The first (black line) and last (grey line) EPSCs recorded during a stimulation train (200Hz, 150ms.) are superimposed to show activity-dependent changes in size, kinetics and timing of synaptic responses. Two analytical approaches, namely the "Threshold Method" (top panel) and "Intersection Method" (bottom panel) are depicted for determination of EPSC onset (arrows). Dotted lines showing the 10% of maximum line are used to determine the onset of EPSCs in the former, whereas dotted lines, fit to the baseline and the 10-90% rise portion of the EPSC, define the intersection point as the onset of EPSCs in the latter. (C) Graphic presentation of the "Maximal Curvature Method" for determination of synaptic delay. The rise phase of an EPSC (dotted line) is first fit with an equation of the form of the Boltzmann charge-voltage equation, the fitted curve is shown (black line). The 4th derivative of the fitted curve is set equal to zero and solved for its three solutions (right panel) of which \( t_1 \) or \( t_3 \) define the maximal curvature point as the EPSC onset.
equation for its 4\textsuperscript{th} derivative (Fig. 4.1C, right panel). Three solutions are generated as shown in Figure 4.1C (and also in Fig. 2.1A, right panel), representing the two points of maximum curvature for the symmetric function (i.e. t\textsubscript{1} and t\textsubscript{2}), and the other being the inflection point (half-maximal time, t\textsubscript{3}). Choosing the solution that occurs earliest in time gives a highly accurate measure of the I\textsubscript{EPSC} onset independent of the slope of the I\textsubscript{EPSC} rise phase (see section 2.5). Such an approach, which we have termed the “Maximal Curvature Method”, reduces intrinsic errors and eliminates potential time biases associated with the other two methods (see Fig. 4.2C,D; also Fig. 2.1D), as is confirmed in the following analyses of representative experiments in which both the amplitude and time course of recorded I\textsubscript{EPSC} exhibit profound changes during repetitive activity.

4.2.2 Validity of the “Maximal Curvature Method” for Analysis of Dynamic Changes in Synaptic Delay during High-Frequency Neurotransmission.

When afferent axons of the calyx of Held synapse were stimulated with high-frequency trains (200 Hz, 150 ms), we observed a robust synaptic depression with I\textsubscript{EPSC} amplitudes declining to about 10% of the initial amplitude near the end of the train, in line with previous reports using similar stimulation protocols (Borst & Sakmann, 1995; Wang & Kaczmarek, 1998; Taschenberger & von Gersdorff, 2000). However, when the first and last events of paired pre- and postsynaptic recordings (30 sweeps superimposed) are contrasted, we noted that the last I\textsubscript{EPSC} appear to have a delayed onset and increased variance in its timing (Fig. 4.2A,B). The small amplitude and highly variable rise phase of the last I\textsubscript{EPSC} in the train made it difficult to accurately quantify SD with the “Threshold Method” or “Intersection Method”. As illustrated in Figure 4.2C & D, when the I\textsubscript{EPSC} rising phase slows, determination of the I\textsubscript{EPSC} onset biases forward in time with the “Threshold Method”, and backward in time with the “Intersection Method”. To evaluate the accuracy of the “Maximal Curvature Method” for determination of the I\textsubscript{EPSC} onset, using the experiment exemplified in Figure 4.2A, we estimated and compared the expected error in the I\textsubscript{EPSC} onset using each of the detection methods (see Fig. 2.1D). Briefly, the linear intersection and maximum curvature methods have similar error for the first event in the train (several \(\mu\)s accuracy), with the error inherent in the threshold method being one order of magnitude larger. For the final event in the train, the linear intersection method showed a 4-fold increase in error (tens of \(\mu\)s accuracy) while the maximum curvature method showed only a modest (~1.5-fold) increase in error (still \(\mu\)s accuracy). The accuracy of the threshold method was unchanged during the train as it is independent of the shape of the I\textsubscript{EPSC} and dependent only on the sampling rate of the digitizer. Both the threshold and linear intersection methods had errors one order of magnitude higher than those of the maximum curvature method for late events. With the I\textsubscript{EPSC} onset depending on the slope of the I\textsubscript{EPSC} rising-phase, for both the linear intersection and threshold methods, we questioned whether either of these methods would bias their determination when the rising-phase of the I\textsubscript{EPSC} slows during repetitive stimulation. Analysis was performed to determine the I\textsubscript{EPSC} onset and associated bias of the first and final events, shown in Figure 4.2A & B, using each of the detection methods. As illustrated in Figure 4.2C (left panel), when the slope of the I\textsubscript{EPSC} rising-phase decreases, the linear intersection method
Figure 4.2: Quantitative Comparison of Different Analytical Methods For Determining Dynamic Changes in the Onset of EPSCs Evoked by High-Frequency Stimuli.

(A) An example of paired presynaptic cell-attached (top panel) and postsynaptic whole-cell voltage-clamp recordings (bottom panel). Thirty current traces, in response to high-frequency afferent stimulation trains (200Hz, 150ms), are superimposed to illustrate activity-dependent changes in synaptic responses. Stimulation artefacts preceding presynaptic and postsynaptic currents have been removed for clarity.  

(B) Magnified view of first (left panel) and final (right panel) stimuli from the traces shown in A. Note the increase in both synaptic delay and variance in events towards the end of trains.  

(C) An example contrasting two EPSCs with fast (black line) and slow rise phases (grey line), for which the “Linear Intersection Method” (left panel) and “Threshold Method” (right panel) both bias the temporal onset of EPSCs.  

(D) Summary data showing the bias inherent in each of the methods shown in C as compared to the “Maximal Curvature Method” for the determination of the EPSC onset.
identifies the point of intersection as occurring earlier in time although the actual $I_{\text{EPSC}}$ onsets may be identical. In contrast, the threshold method biases the $I_{\text{EPSC}}$ onset forward in time, relative to $I_{\text{EPSC}}$ with steep rising-phases. Using each of the methods to calculate the position of the $I_{\text{EPSC}}$ onset from the traces shown in Figure 4.2A, we find that the Threshold method biased and average of $7 \pm 1\%$ forward in time for first and $7 \pm 3\%$ for final $I_{\text{EPSC}}$, while the linear intersection method biased backwards in time an average of $3 \pm 1\%$ and $8 \pm 4\%$ for first and final $I_{\text{EPSC}}$ respectively, as compared to the maximum curvature method (Fig. 4.2D). While the bias averages to small discrepancies in the $I_{\text{EPSC}}$ onset, these discrepancies vary from 2 to 20\% and $-24$ to 44\% for the threshold method (first and last events respectively) for some traces. The linear intersection method showing a range of discrepancies of $-9$ to $11\%$ and $-61$ to 31\%, for first and last events respectively, over the 30 sweeps analyzed. When both the error and methodological bias are considered, in the context of delay changes of generally less than 500$\mu$s during high-frequency transmission, it is evident that these methods are insufficient to resolve small changes in delay from event to event. Furthermore, bias, depending on the detection method chosen, may mask or enhance small changes in delay, producing misleading results. Given the results of such comparative analyses, we suggest that the “Maximal Curvature Method” provides a significant advantage over previous methods and can measure small changes in the various temporal components of synaptic transmission with accuracy on the order of microseconds throughout stimulus trains.

4.2.3 Frequency-Dependent Prolongation in Transmission Delays & Variance.

Inspection of pre- and postsynaptic recordings reveal that, in contrast to the profound jitter of the last $I_{\text{EPSC}}$, current traces reflecting presynaptic APs show little variation in shape and size between sweeps (Fig. 4.2B, right panel), suggesting that activity-dependent changes in SD and variance occur downstream of AP arrival at the calyx terminal. Armed with our novel analytical approach, we next quantified three temporal components, namely CD, SD and RRD, of synaptic transmission. Thirty sweeps of pre- and postsynaptic events, in response to stimulus trains at 20, 50, 100, and 200Hz (30 stimuli per train), were recorded and analyzed for each experimental condition designed to explore the potential mechanisms underlying these short-term temporal changes in delay components.

Figure 4.3A illustrates an example of paired pre- and postsynaptic recordings in response to 30 sweeps of train stimuli at various frequencies in 2mM extracellular Ca$^{2+}$ ([Ca$^{2+}$]$_o$). Sweeps from the 1$^{\text{st}}$ (black) and 30$^{\text{th}}$ (grey) events are superimposed. Initial events from this recording were found to have basal TD (the sum of the three component delays) of approximately 1.34ms, with CD, SD and RRD comprising about 0.44, 0.51 and 0.39ms, respectively (Fig. 4.3A,D). When these temporal components were quantified from each pair of events, and plotted against the stimulus number, we found that all three delays were prolonged by about 5-30\% in a frequency-dependent manner, being most robust at 200Hz (Fig. 4.3B). These changes were well described by a linear function with a slope or rate of delay progression ($s, \mu s \cdot \text{event}^{-1}$), the values of which are summarized in Table 4.1A for all delay components and frequencies tested (Fig. 4.3C, top panel). In parallel, these delays also showed frequency-dependent increases in their variance (in $\mu s^2 \cdot \text{event}^{-1}$), which also followed a linear functions with slope
Figure 4.3: Frequency-Dependence of Increases in Neurotransmission Delay & Variance During Repetitive Stimulation.

(A) First (black) and 30th (grey) pairs of presynaptic current deflections and postsynaptic EPSCs, from thirty repeated stimulation trains at 20Hz (left panel), 50Hz (middle panel), and 100Hz (right panel), are aligned and superimposed to illustrate the frequency-dependence of delay in pre and postsynaptic 2+ responses (P15, 2mM [Ca2+]).

(B) Plots illustrating the change in SD (top panel), RRD (middle panel), and TD (bottom panel), as a function of stimulus number, for 20Hz (green), 50Hz (blue), 100Hz (red), and 200Hz (black) stimulation frequencies shown in A. Data shown for 200Hz taken from example shown in Fig. 4.2A. Dashed lines are least-squares regressions through data shown.

(C) Summary data showing the progression of delay (μs·event−1, top) or variance (μs2·event−1, bottom) for each delay component at each frequency shown in B. (D) Summary data showing the percent change in delay and variance, compared between the first and final three events in a train, for each delay component at each frequency shown in B. Asterisks denote significance (p<0.05) against 200Hz group (black bars).
### Table 4.1: Summary Data Values for Experiments Shown in Figure 4.3.

(A) Progression rate of delay components as a function of stimulation frequency.  
(B) As in A showing the progression of variance.  
(C) Percent change in delay components as a function of stimulation frequency.  
(D) As in C showing the percent change in variance.
values summarized in Table 4.1B for all delay components and frequencies tested (Fig. 4.3C, bottom panel). Furthermore, we calculated the percent change in both delay and variance for each of the stimulation frequencies tested. Summary data values are shown in Table 4.1 C and D for percent change in delay and variance respectively for each stimulation frequency tested (Fig. 4.3D, top & bottom panels). As a result of prolongations in individual delay components and their variance, TD and its variance displayed significant increases, as illustrated in Figure 4.3C & D (see Table 4.3).

The magnitude of activity-dependent prolongation in SD, RD, and therefore TD, depend strongly on the frequency of the activity with stronger activity evoking more prolongations in delays and increases in variance. Our observations at the calyx of Held synapse demonstrate that the temporal components of synaptic transmission, including SD, are highly sensitive to activity and can undergo short-term plastic changes in central synapses.

4.2.4 Lowering Ca²⁺ Influx into the Nerve Terminal Reduces Activity-Dependent Increases in Transmission Delays and Variance.

Given that the calyx of Held synapse exhibits the most profound changes in temporal delay components at 200Hz, where synaptic depression is also strongest over the tested frequency spectrum, we asked whether reducing the extent of synaptic depression can attenuate temporal changes in delays and their variances. To this end, we reduced [Ca²⁺]₀ from 2mM to 1mM, thereby reducing the driving force for Ca²⁺ entering the nerve terminal and attenuating synaptic depression during high-frequency stimulation. Overlaying the 1⁰ and 30⁰ pairs of events, as in Figure 4.4A & B, shows that neither the size nor the shape of presynaptic APs was significantly affected by lowering [Ca²⁺]₀ but initial synaptic strength and synaptic depression were reduced as expected. Analyses of the various temporal delays reveals that the activity-dependent increase in CD was not affected by lowering [Ca²⁺]₀ while the progression of SD, RRD, and TD, as well as their variances, decreased significantly (p<0.05). Pooled values for progression of delay and variance for the tested Ca²⁺ concentrations are shown in Table 4.2A & B respectively (Fig. 4.4C,D, also see Fig. 4.8).

If we compare the percent increase, in either delay or variance, between the 1⁰ and 30⁰ events, lowering [Ca²⁺]₀ from 2mM to 1mM reduced the increase in SD, RRD, and TD delay and variance significantly (Table 4.2C,D, Fig. 4.8). Neither the delay nor variance of the conduction component was affected by altering [Ca²⁺]₀ (Fig. 4.4C,D, top panels; Fig. 4.8). In line with these results, we found that the progression rate of delays, and the increase in their variances, were reduced in 2mM [Ca²⁺]₀ with the addition of 50μM of the GABA₉R agonist baclofen (Table 4.2A,B, Fig. 4.8 & 9.1.1), which acts through a G-protein mediated signalling cascade (Gβγ) to inhibit P/Q-type Ca²⁺ channels (Isaacson, 1998; Takahashi et al., 1998; Kajikawa et al., 2001), the type predominantly mediating release of neurotransmitter in the calyx of Held at this developmental stage (Forsythe et al., 1998; Iwasaki et al., 2000). These data collectively suggest that a reduction in the influx of Ca²⁺ into the nerve terminal attenuates the rate and degree to which delay and variance increase during high-frequency synaptic activity.
Figure 4.4: Activity- & Ca\textsuperscript{2+}-Dependence of Neurotransmission Delay Components.

(A-B) First (black lines) and last (grey lines) pairs of presynaptic current deflections and postsynaptic EPSCs from thirty repeated stimulation trains (200Hz, 150ms) are aligned and superimposed to illustrate activity-dependent changes in presynaptic and postsynaptic responses in 2mM [Ca\textsuperscript{2+}] (A) and 1mM [Ca\textsuperscript{2+}] (B).  (C-D) Plots for each measurable delay component against the number of stimuli in a train under the two experimental conditions described in A & B. Lines in these plots represent linear regression to scattered data points of thirty sweeps.
4.2.5 Increasing Temperature Reduces Activity-Dependent Increases in Transmission Delays & Variance.

Because elevating temperature shortens presynaptic AP width, reduces Ca\(^{2+}\) influx and short-term depression during train stimuli (Taschenberger & von Gersdorff, 2000; Kushmerick et al., 2006; Yang & Wang 2006), we next examined activity-dependent changes in delay for the three temporal components, and their variances, at the near physiological temperature of 35\(^{\circ}\)C in 2mM [Ca\(^{2+}\)]\(_o\). At 200Hz, we found that synaptic transmission was accelerated with all temporal delay components shortened at the onset of the stimulus train (i.e. 1\(^{st}\) events). Activity-dependent increases in delays, and their variances, remained, though to a lesser extent than that at room temperature (Fig. 4.5A,C vs. Fig. 4.4A,C; Fig. 4.8). SD, RRD and TD progressed during the train at the rates shown in Table 4.2A, while the variance of these components also increased at the rates shown in Table 4.2B. The extent to which the delay and variance increased in 2mM [Ca\(^{2+}\)]\(_o\) at 35\(^{\circ}\)C was similar to that recorded at room temperature in 1mM [Ca\(^{2+}\)]\(_o\) (Table 4.2C,D). However, when we increased the stimulation frequency to 400Hz at 35\(^{\circ}\)C, prolongation in delay components and their variance again increased markedly, with progression and variance prolongation rates shown in Table 4.2A & B (Fig. 4.5B,D). The percent increase in delay and variance for these delay components showed similar increases when stimulation frequency was increased at 35\(^{\circ}\)C (Table 4.2C,D). In contrast, CD and its variance were unaltered by such a manipulation (Table 4.2C,D, Fig. 4.8). These data suggest that activity-dependent increases in temporal delays and the propensity for temporal jitter, during high frequency stimulation, are intrinsic physiological properties of this synapse and can occur at near physiological temperatures.

4.2.6 Prolongation in Delay & Increase in Variance Occurs Downstream of Ca\(^{2+}\) Influx.

Although high-frequency train stimulation led to an increase in CD, presynaptic recordings showed that the 30\(^{th}\) APs displayed little variation in their waveform or temporal jitter (e.g. Fig. 4.2B), implying that the prolongation in SD and RRD, and progressive increases in their variance, must originate downstream of APs themselves. One source of such changes may be that the size and timing of Ca\(^{2+}\) currents evoked by AP trains fluctuates between sweeps, introducing variations in quantal output and temporal asynchrony of fusion events. To test this, we performed whole-cell voltage-clamp recordings of Ca\(^{2+}\) currents from the calyx terminal, for which we have previously demonstrated that good space-clamp can be achieved (Yang & Wang, 2006). Ca\(^{2+}\) currents were evoked in response to a train of real APs used as a voltage-clamp command waveform. These APs were previously recorded from a current-clamped calyx (P15) in response to axonal stimulation at 200 Hz for 200 ms (Fig. 4.6A). Ca\(^{2+}\) currents were then examined for fidelity in amplitude and timing. Figure 4.6B shows recordings of ten superimposed traces of Ca\(^{2+}\) currents evoked, as above, in 2mM [Ca\(^{2+}\)]\(_o\) (left panel), 1mM [Ca\(^{2+}\)]\(_o\) (middle panel), and 2mM [Ca\(^{2+}\)]\(_o\) with 50\(\mu\)M baclofen (right panel). Although the amplitude and facilitation of Ca\(^{2+}\) currents differed in the three conditions, their shape and size exhibit extremely high fidelity during repeated sweeps, with no observable jitter in the timing at any stage during the high-frequency AP trains (Fig. 4.6B, insets). These observations were highly reproducible among different
Figure 4.5: Temperature-Dependence of Neurotransmission Delay Components.

(A-B) First (black lines) and last (grey lines) pairs of presynaptic current deflections and postsynaptic EPSCs from thirty repeated stimulation trains at 200Hz (A) and 400Hz (B) are aligned and superimposed to illustrate the frequency-dependence of changes in presynaptic and postsynaptic responses at 35°C. Note that time scales are different in A & B.

(C-D) Plots for each measurable delay component against the number of stimuli in a train under the two experimental conditions described in A & B. Lines in these plots represent linear regression to scattered data points of thirty sweeps.
Figure 4.6: Temporal Plasticity of Synaptic Transmission is Mediated by Mechanisms Downstream of AP Evoked Ca\(^{2+}\) Influx.

(A) Voltage-clamp template of an AP train (200Hz, 200ms) used for evoking Ca\(^{2+}\) currents from calyces. Note that these real APs were previously recorded in whole-cell current-clamp configuration from a nerve terminal (P15), in response to afferent stimulation, and digitally converted into a voltage-clamp command file (See Methodology 2.2.5). (B) Ca\(^{2+}\) currents evoked by the AP train template in 2mM [Ca\(^{2+}\)], (left panel), 1mM [Ca\(^{2+}\)], (middle panel), and 2mM [Ca\(^{2+}\)] w/50μM baclofen (right panel) solutions are shown. In each case, ten repeated sweeps of Ca\(^{2+}\) currents are overlaid to demonstrate their temporal fidelity. Insets (B, bottom panel) contrast the first and last two Ca\(^{2+}\) currents magnified from those shown in the top panels.
calyces (n=5-10 for each condition). The fact that AP-evoked Ca^{2+} currents display such high-fidelity suggests that the source of the observed activity-dependent increases in delay and variance occurs downstream of Ca^{2+} channel gating.

### 4.2.7 Reducing Intra-Terminal Ca^{2+} Accumulation Attenuates Activity-Dependent Increases in Delay & Variance.

Knowing that Ca^{2+} currents preserve fidelity in their timing during high-frequency synaptic transmission, we next asked whether accumulation of residual Ca^{2+}, during repetitive activity, underlies the observed activity-dependent temporal plasticity. To investigate this possibility, we made use of EGTA-AM, a membrane permeable Ca^{2+} buffer, which accumulates intracellularly following cleavage of its AM-ester group by endogenous esterases. Because EGTA has a slow forward rate constant for the binding of Ca^{2+} ions, it is assumed that it may effectively buffer accumulation of residual Ca^{2+} in the terminal (Alder et al., 1991; Kamiya & Zucker, 1994; Regehr et al., 1994; Naraghi & Neher, 1997, Fedchyshyn & Wang, 2005). Although it is difficult to know the exact amount of buffer that has accumulated in the presynaptic terminal, following pretreatment of slices with EGTA-AM (50 μM) for 30 min, we noted that the amplitude of I_{EPSC} were noticeably attenuated from 6.1±0.6 to 4.0±0.6 nA (Fig. 4.7A,B, top panel), consistent with previous observation that EGTA can block a fraction of transmitter release either with direct loading into the terminal through patch pipette or incubation of AM-ester form for synapses at the developmental stage used for this study (Fedchyshyn & Wang, 2005; Korogod et al. 2005). Furthermore, EGTA-AM pretreatment also led to a slight facilitation in I_{EPSC} while the extent of synaptic depression was reduced and its kinetics slowed, in line with the effects of this buffer at the nerve terminal (Fig. 4.7B, bottom panel). In pretreated synapses, high-frequency stimulation of afferent axons resulted in attenuated progression rates for SD, RRD, and TD (Table 4.2A, Fig. 4.7C). The extent to which delay increased was also reduced for all delays (Table 4.2C). In parallel, the progression rate of variance for each delay component was also attenuated by pre-treatment with EGTA-AM (Table 4.2B). Similarly, the extent of increase in the variance declined following EGTA-AM pre-treatment (Table 4.2D, Fig. 4.8). These observations demonstrate that activity-dependent changes in delays, and their variances, strongly depend on the build-up of residual Ca^{2+} in the calyx terminal. Since APs and Ca^{2+} currents precisely preserve timing during repetitive activity, we suggest that this build-up underlies the breakdown of synchrony and reproducibility of fusion events.

### 4.2.8 Mechanisms Underlying Activity-Dependent Prolongation in Temporal Delays.

Reducing Ca^{2+} influx into the calyx or buffering intra-terminal accumulation of Ca^{2+} in the aforementioned experiments all led to decreases in activity-dependent prolongation of temporal delays of transmission and their variances, as well as the extent of short-term synaptic depression in synaptic strength. Given these observations, we hypothesized that changes in synaptic strength and temporal delays during high-frequency activity may be related. To test this, we measured the recovery time course of synaptic strength and delay components by delivering single test pulses at different time
Figure 4.7: Intra-Terminal Accumulation of Residual Ca\(^{2+}\) Underlies Activity-Dependent Prolongation in Neurotransmission Delay Components.

(A) Examples of first (black lines) and last (grey lines) pairs of presynaptic current deflections and postsynaptic EPSCs from thirty repeated stimulation trains (200Hz, 150ms) in 2mM [Ca\(^{2+}\)]\(_o\) w/EGTA-AM pre-treatment. (B) Pooled data showing the amplitude of the first EPSCs (top panel) and a plot of normalized EPSC amplitude against event number in 2mM [Ca\(^{2+}\)]\(_o\) w/EGTA-AM pre-treatment (bottom panel). (C) Plots for delay components against the number of stimuli in a train under the experimental conditions described in A. Solid black lines represent linear regression to scattered data points shown. Dashed lines represent linear regression to scattered data shown in Fig. 4.4C.
Figure 4.8: Comparison of Activity-Dependent Changes in Temporal Delays & Their Variance. (A-B) Pooled data showing the progression rate of delay (A) for all temporal components and their variance (B) for various experimental conditions. (C-D) Pooled data showing the relative extent of the prolongation in various delay components (C) and their variance (D), as in A and B. Asterisk shows significance (p<0.05, Student’s t-test) against the 2mM [Ca\(^{2+}\)] data set. Comparisons between other data sets are shown in Table 4.1.
Table 4.2: Summary Data Values for Experiments Shown in Figures 4.4 to 4.8.

(A) Progression rate of delay components as a function of experimental condition. (B) As in A showing the progression of variance. (C) Percent change in delay components as a function of experimental condition. (D) As in C showing the percent change in variance.

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<th>Synaptic Delay (μs/event)</th>
<th>Response-Rise Delay (μs/event)</th>
<th>Transmission Delay (ms/event)</th>
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intervals (Δt) after repeated high-frequency trains (Fig. 4.9A,B). Figure 4.9A shows a typical experiment in which the amplitude of I_{EPSC} declined in response to the test train (200Hz, 150ms), but gradually recovered in as Δt increased. When pre- and postsynaptic responses, evoked by single test pulses at different intervals, were superimposed and aligned by stimulus artefact (Fig. 4.9B), we noted that the temporal delays recovered in parallel with the recovery of synaptic strength (Fig. 4.9C,D). Recovery of the delay components showed that CD, SD, and TD all followed a biphasic exponential time-course with fast and slow time constants ranging from 13 to 82ms and 1100 to 2300ms, respectively. Recovery of RRD was fit with single exponential function (τ=170 ms) as RRD data appeared to be scattered, likely due to significant asynchrony of quantal release and small amplitude of I_{EPSC} (Fig. 4.9D). These values are similar to those for the recovery time-course of synaptic strength (Fig. 4.9C). Plotting the recovery of SD (Fig. 4.9E, left panel) and TD (Fig. 4.9D, right panel) against the recovery of I_{EPSC} yielded linear correlations significantly past 95% confidence (R=0.82 and 0.87 for SD and RD respectively) and suggest that the recovery of both delay and I_{EPSC} may share an underlying mechanism. Upon closer inspection of our data on changes in SD, RD and TD, we noted that prolongations in these parameters and their variance became most apparent when the amplitude of I_{EPSC}, during high-frequency train, was depressed to about 30% of the initial quantal output (e.g. Fig. 4.4C). Furthermore, when the amplitude of the I_{EPSC} evoked by the test pulse returned to about 30% of the first I_{EPSC}, the fast component of recovery for prolongations in SD, RD and TD was also complete. We interpreted these lines of evidence to suggest that high-frequency activity leads to a depletion of the readily-releasable pool of SVs, and/or a reduction of release probability. Depletion transforms transmitter release from fast synchronous events, near the beginning of the train, to slower asynchronous, stochastic events towards the end. Following the cessation of a train, replenishment of depleted release sites with SVs from the peripheral reserve pool at least partially restored synaptic strength and temporal fidelity of fusion events.
Figure 4.9: Recovery of Delay Components From Activity-Dependent Prolongation.

(A) Stimulation protocol (top) for determining the recovery kinetics of delay components. 200Hz, 150ms trains were followed by a single test pulse at varying Δt from 10-1500ms. Example traces showing averaged presynaptic current deflections (middle panel) and postsynaptic I (bottom panel), in EPSC response to the stimulation protocol in A, for Δt=10ms, 500ms, 1000ms, and 1500ms. (B) Magnification of the events shown in A are aligned by presynaptic stimulation artefact for the Δt in A (as marked). (C) Pooled data plotting I_{EPSC} area, as a percent of the initial I_{EPSC} area, against Δt. Points were fitted with a biphasic exponential (solid line). (D) Pooled data plotting delays, as a percent of the initial delay, against Δt. Points were fitted with a biphasic exponential (solid lines) in all cases except for response delay which was fitted with a single exponential. (E) Correlation plots of I_{EPSC} recovery vs. recovery of SD (left panel) and TD (right panel) as a percentage of the initial events. Solid black lines represent linear regression to scattered data points shown.
4.3 DISCUSSION

With a novel and unbiased analytic approach, termed the "Maximal Curvature Method", to precisely dissect out different delay components of synaptic transmission (Fig. 4.1 & 2.1), we have revealed a new form of activity-dependent, short-term plasticity at the calyx of Held synapse. We found that high frequency axonal stimulation prolongs the various temporal components underlying SV release and increases their variance in a frequency-dependent manner (Fig. 4.3). We have further demonstrated that such changes at 200Hz can be attenuated by reducing the amount of Ca^{2+} influx via lowering [Ca^{2+}]_o, raising experimental temperature, or inhibiting VGCCs with GABA_B agonist baclofen (Fig. 4.4, 4.5 & 10.1.1). Changes in delays and their variance are independent of presynaptic I_{Ca} (Fig. 4.6), but facilitation of I_{Ca} during a train of APs may contribute to intra-terminal accumulation of residual Ca^{2+}. As calyceal preloading of the slow Ca^{2+} buffer EGTA-AM effectively reduces the progression rate of activity-dependent increases in various delay components and their variances, we suggest that the build-up of residual Ca^{2+} downstream of Ca^{2+} entry during APs is likely the key mediator of such changes (Fig. 4.7). A quantitative description of the various temporal delays and their variances under different experimental conditions are presented in Figure 4.8. Both the slope, representing the progression rate in each component delay and its variance (Fig. 4.8A,B) and the relative increase in each delay and variance component, from the beginning to the end of test train (i.e. 1st vs. 30th event) (Fig. 4.8C,D) are compared. These results present compelling evidence that central synapses can exhibit temporal changes in the form of activity and Ca^{2+}-dependent increases in delay and variance, along with changes in synaptic strength.

Among the three delay components that we have separated, with paired recordings from pre- and postsynaptic elements of the same synapses, CD shows similar prolongation to the other delay components but little change in its delay or variance under most experimental conditions (Fig. 4.8). This is evident from the observation that the presynaptic AP waveform remains highly superimposed, between sweeps and throughout the trains (e.g. Fig. 4.2B), despite slight AP broadening during each stimulation train (i.e. increased peak-to peak time interval between inward and outward current deflections in presynaptic cell-attached recordings). Large axons leading to the calyceal terminals presumably play an important role in maintaining temporal fidelity in CD. Nevertheless, absolute time for CD can be significantly shortened by increasing temperature, but activity-dependent prolongation in CD remains and is resistant to experimental manipulations. It is possible that axons still experience fatigue due to the build-up of K^+ ions and/or undergo Na^+ channel inactivation during repetitive high-frequency activity, or a process sensitive to temperature but not Ca^{2+}, as suggested by our observations under different conditions in this study.

In contrast to CD, SD and RRD are highly sensitive to manipulations that affect intra-terminal Ca^{2+} levels. By lowering [Ca^{2+}]_o, or attenuating Ca^{2+} currents with baclofen, activity-dependent prolongation in these two delays and their variances decreased significantly, suggesting that temporal changes in these parameters are Ca^{2+}-dependent. Direct voltage-clamp recordings of Ca^{2+} currents, in response to repeated trains of high-frequency APs, revealed that these currents are highly faithful in
timing and waveform, indicating that the progressive increase and fluctuations in the latency of $I_{EPSC}$ originates downstream of $Ca^{2+}$ entry. Indeed, our observation that EGTA-AM pretreatment attenuates activity-dependent increases in SD and RRD, and their variances, suggests that residual accumulation of intra-terminal $Ca^{2+}$ is a critical factor underlying such temporal plasticity. Because conditions which influence the rate and extent to which synaptic depression occurs also appear to influence the rate of progression of delay and variance (e.g. $[Ca^{2+}]_o$), we suggest that both these forms of short-term plasticity may share common underlying mechanisms. Indeed, recovery kinetics in synaptic strength, following synaptic depression by high-frequency trains, appears to correlate with those of SD and TD (Fig. 4.9).

We interpret these observations such that high frequency activity sequentially depletes the readily releasable pool (RRP) of SVs, which are coupled most tightly to release sites and are released with the shortest latencies. Release of SVs, recruited from the periphery of the RRP, follows this rapid release (Wang & Kaczmarek, 1998; Sakaba & Neher, 2001; Schneggenburger et al., 2002; Otsu et al., 2004; Sakaba, 2006). Because of the heterogeneous distance of peripheral SVs to the release sites, the increased time required for the recruitment and release of these SVs may therefore introduce not only prolongations in SD but also variability or asynchrony in the timing of release events. Increased jitter in SD towards the later part of trains may simply mirror the inherent stochastic nature of vesicular release when tightly docked vesicles are depleted and/or release probability is substantially reduced, as has been demonstrated at the endbulb of Held synapse with $Ca^{2+}$ channel blocker cadmium (Isaacson & Walmsley, 1995). However, we cannot exclude the possibility that temporal changes in SD and RRD could result from an activity-dependent refractory period of release sites (Stevens & Wang, 1995) or exhaustion of the release machinery (e.g. $Ca^{2+}$ sensor) (Hsu et al., 1996), leading to short-term transient prolongations in temporal delays.

Previous work has shown that first latency distribution histograms of release events at the NMJ display remarkable stability under different experimental conditions, implying that the minimal SD is rather static and therefore the timing of exocytosis following AP invasion is not plastic (Katz & Miledi, 1965; Barrett & Stevens, 1972; Datyner & Gage, 1980; Parnas et al., 1989). However, these studies were done primarily with single or pairs of stimuli. Little is known regarding whether the various temporal components of neurotransmission show activity-dependent plasticity when synapses are presented with more physiological stimuli such as high frequency trains. Indeed, when this issue was investigated in non-mammalian synapses using other paradigms such as paired-pulse stimuli or trains, it was demonstrated that SD could be modified under appropriate conditions (Vysheskiky et al., 2000; Waldeck et al., 2000). At the calyx of Held-MNTB synapse, increases in both AP latency and $I_{EPSC}$ onset have also been observed following prolonged tetanic stimulation (Habets & Borst, 2005; Kim et al., 2007). However, it is not know whether such changes are due to changes in the timing and magnitude of $Ca^{2+}$ currents or depend on factors residing downstream of $Ca^{2+}$ entry. Our study has clearly demonstrated that increases in SD and its variance are both a result of residual $Ca^{2+}$ accumulation downstream of AP-evoked $Ca^{2+}$ influx during repetitive activity. Interestingly, Wu and Borst (1999) showed in the immature calyx of Held synapses (P8-10), that SD, in response to a pair of brief APs (half-width: 0.5ms, amplitude: -80mV to +30mV), was not altered during synaptic depression. However, SD was prolonged when
assayed with a prolonged pair of voltage steps with longer duration (10ms), and of smaller amplitude (-80mV to 0mV), than real APs. This observation lends support to our interpretation that activity-dependent increases in SD and its variance reflect sequential depletion and recruitment of SVs during train stimuli. High-frequency activity mimics the effects of prolonged steps, where the first step largely depletes the SVs in the RRP and the second step recruits latent peripheral SVs to the depleted release sites increasing the SD.

By paired recordings directly from the nerve terminal and its postsynaptic target neuron, we have provided insights into understanding the constraints of temporal fidelity of neurotransmission. We have also provided the proof-in-principle that activity-dependent changes in the temporal delay components of synaptic transmission can occur in mammalian central synapses. One can envision that such temporal changes may be common and more profound for typical en passant synapses with thin axons and tiny boutons containing limited number of SVs. AP propagation along thin axons may experience significant increase in CD during repetitive activity while small size of readily-releasable pool of SVs may be depleted quickly to cause prolongations in SD and RD. Furthermore, presynaptic boutons are spatially innervating different parts of dendrite branches of the same target neuron, potentially expanding the window for temporal summation of inputs. Temporal delays in such circumstances may in fact be of physiological significance for neural computation (Markram et al., 1998; Lu & Trussell, 1997). Being a critical relay station specialized in preserving timing information for sound localization, the calyx of Held-MNTB synapse must be superior to other synapses in preserving the temporal fidelity of synaptic input. Our experiments at near physiological temperature of 35°C demonstrate that temporal changes in three delay components is indeed limited at 200Hz but exacerbate with an increase in stimulation frequency (e.g. 400Hz), indicating that such changes are inherent properties of synapses. Nevertheless, knowing that this synapse can operate at much higher frequencies (>600Hz), our observations lead us to suggest that there are several important strategic adaptations, which the calyx of Held synapse may develop for best preserving timing information. First, we have demonstrated in this study that synaptic depression and prolongations in temporal delays are two well-correlated processes (Fig. 4.9). Developmental narrowing APs at the calyx would make Ca$^{2+}$ transients very brief and reduce the extent of short-term depression in synaptic strength during high-frequency transmission, thereby minimizing delays and maximizing their reproducibility. Such effects are reminiscent of those by shortening the half-width of APs at higher temperature (Fedchyshyn & Wang 2005; Kushmerick et al., 2006; Yang & Wang, 2006). Second, efficient clearance of Ca$^{2+}$ by buffering and extrusion may help reduce the effects of residual Ca$^{2+}$ build-up on prolongations in delays, as implicated by our EGTA-AM experiments (Fig. 4.7). In fact, developmental up-regulation of endogenous calyceal Ca$^{2+}$ buffering proteins, such calretinin and parvalbumin, beyond the postnatal stage used in this study (i.e. P14-16), has been demonstrated (Felmy & Schneggenburger, 2004). Ca$^{2+}$ pumps/exchangers may also operate more efficiently at physiological temperature to accelerate removal of residual Ca$^{2+}$ (Helmchen et al., 1997). Finally, it should also be noted that although 2mM [Ca$^{2+}$]o is generally used in a majority of studies performed in brain slices, physiological [Ca$^{2+}$]o is likely closer to 1mM (Jones & Keep, 1988). Lower [Ca$^{2+}$]o can reduce the driving force for Ca$^{2+}$ influx during each spike, and further minimize the temporal changes of neurotransmission
as observed in this study (Fig. 4.4). Taken together, these factors may be potentially crucial for curtailing accumulation of intra-terminal [Ca^{2+}], and preserving temporal fidelity of synaptic transmission at the calyx of Held synapse.
**Table 4.3:** Cross Examination of Statistical Significance Between Different Experimental Conditions.

(A) Table summarizing statistical significance (p<0.05, Student's t-test) between various stimulation frequencies for progression of delay and variance. Delay comparisons are shown in blue, variance comparisons are shown in green. NS signifies no significant difference. (B) As in A comparing percent change in delay and variance. Column labels refer to the stimulation frequency during experiments. (C) Table summarizing statistical significance between various experimental conditions for progression of delay and variance. (D) As in C comparing percent change in delay and variance. Column labels 1mM, 2mM, EGTA, BacI, 35C, and 400HZ refer to experiments conducted in 1mM [Ca^{2+}], 2mM [Ca^{2+}], 2mM [Ca^{2+}], with 50μM EGTA-AM pre-treatment, 2mM [Ca^{2+}], with 50μM Baclofen, 2mM [Ca^{2+}], at 35°C with trains at 200Hz, and 2mM [Ca^{2+}], at 35°C with trains at 400Hz respectively.
5. THE SEPTIN 5 PROTEIN IS A MOLECULAR DETERMINANT OF CALCIUM CHANNEL-SYNAPTIC VESICLE SPATIAL COUPLING AT THE DEVELOPING CALYX OF HELD SYNAPSE
5.1 INTRODUCTION

During development, central synapses undergo a number of pre- and postsynaptic modifications which confer their ability to perform certain processing tasks more effectively (Li & Sheng, 2003). The signalling events that trigger these changes in synaptic properties can arise from genetic programming, sensory/synaptic input, or particular combinations of both, and result in specific and compartmentalized alterations to any of the steps in neuronal signal transduction. While much insight has been gained as to how the postsynaptic element of a synapse develops in response to various forms of activity (i.e. tetanic stimulation and LTP), the elements of presynaptic development have been more difficult to characterize directly (Chuma & Ohmori, 1998; Sheng & Kim, 2002; McGee & Bredt, 2003; Takahashi, 2005; Tada & Sheng, 2005). The relatively recent development of the calyx of Held-MNTB synapse preparation, with its giant presynaptic terminal, has facilitated direct study of mammalian central nerve terminals previously inaccessible due to their prohibitively small size (Forsythe, 1994; Borst et al., 1995). This synapse, located in the auditory brainstem and crucial for the localization of sound, is ideal for the study of presynaptic development of synaptic transmission as; it is accessible to whole-cell electrophysiological recordings, the pre- and post synaptic elements form a monosynaptic connection, it develops mature functionality over a short and well-characterized period, and is specialized to reproduce high-frequency input signals with high fidelity (Trussell, 1999; Taschenberger & von Gersdorff, 2000; von Gersdorff & Borst, 2002).

In previous chapters, we characterized a novel presynaptic developmental adaptation, occurring at the approximate time of hearing onset (~P11), in which the spatial separation between voltage-gated calcium channels (VGCCs) and synaptic vesicles (SVs) decreased with synaptic maturation (Fedchyshyn & Wang, 2005). At the calyx of Held, immature synapses (P8-12) employ a heterogeneous arrangement of loosely-coupled VGCCs, of both N- and P/Q-types, to provide the Ca\(^{2+}\) signal necessary to trigger release of a SV (“microdomain” modality). Mature synapses, in contrast, release vesicles with a tightly-coupled array VGCCs, of the P/Q-type exclusively, to perform the same task (“nanodomain modality”). The by-product of this change in release modality is that mature synapses require significantly fewer (as few as 1) Ca\(^{2+}\) domains to trigger release of a SV than do immature synapses (Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005). This phenomenon is observed as a significant decrease in the degree of non-linearity of the synaptic input-output relationship, defined as “Ca\(^{2+}\)-domain cooperativity” or the exponent \(m\) from \(I_{\text{EPSC}} \propto [I_{\text{Ca}}]^m\), during development (Hill, 1910; Dodge & Rahamimoff, 1967; Gentile & Stanley, 2004; Fedchyshyn & Wang, 2005). In addition, tightening of VGCC-SV coupling works, in opposition to developmental decreases in action potential (AP) width, to maintain synaptic efficacy and release probability (P\(_r\)) (Yang & Wang, 2006).

While it is clear that changes in VGCC-SV coupling will have profound effects on the P\(_r\) of a SV, for a given input/Ca\(^{2+}\) current (\(I_{\text{Ca}}\)), it is not clear whether these same coupling changes also impact the timing of SV release. In fact, over many decades there has been controversy as to whether factors altering P\(_r\) have any effect at all on the latency distribution of released quanta (Katz & Miledi, 1965; Barrett & Stevens, 1972; Datyner & Gage; 1980; Parnas et al., 1989; Waldeck et al., 2000; Vyshedskiy et
Developmental decreases in synaptic delay (SD), the time between innervation of a presynaptic AP and the onset of a postsynaptic response, have been characterized at the calyx of Held synapse previously; however, whether the decrease in SD is the result of changes in the kinetics of presynaptic APs or occur further downstream is not clear (Taschenberger & von Gersdorff, 2000, Taschenberger et al., 2005). In addition, SD appears to also depend on the magnitude of $I_{\text{Ca}}$, as increases in $[\text{Ca}^{2+}]_o$ result in shortened SD at the immature calyx of Held (Borst & Sakmann, 1996). Conversely, decreases in $P_r$, brought about through depletion of the readily releasable pool (RRP) of SVs, results in an increase in SD, although this is only measurable in response to prolonged presynaptic voltage steps or bouts of high-frequency activity (Wu & Borst, 1999; Fedchyshyn & Wang, 2007). These findings raise the possibility that alterations to the size and spread of Ca$^{2+}$-domains can influence the temporal profile of SV release depending on the spatial intimacy of VGCCs and SVs.

While the effects of changes in VGCC-SV coupling are becoming better understood, at least in terms of synaptic output, the molecular mechanisms underlying this change are not known. The presynaptic cytomatrix of the active zone (CAZ) is comprised of a number of recently identified interacting proteins that serve to organize, dock, and prime SVs for release (reviewed in Schoch & Gundelfinger, 2006; Dresbach et al., 2001). A filamentous mesh of projections emanating from the AZ appear to encircle those SVs in close proximity to the presynaptic membrane, while filaments can also be seen in the space between SVs located further from the AZ (Hirokawa et al., 1989). These strands that link SVs to actin filaments, microtubules, and to each other, are thought to be comprised of the synapsins; however, the composition of those connecting SVs to the AZ remain unknown (Hirokawa et al., 1989; Beites et al., 2005).

Septin 5 (Sept5) is a filamentous protein that has been found associated with SVs through its interaction with syntaxin and has been hypothesized to act as a molecular “brake” preventing SV association with the t-SNARE complex. The mechanism for this action has not been determined but Sept5 filaments, or Sept5 containing filaments, are hypothesized to either bind to 7S SNARE complexes between the AZ and SVs or to 7S complexes across the AZ inhibiting release (Beites et al., 1999; Beites et al., 2005). Sept5 can bind syntaxin, either alone or when it is part of the 7S complex, at either end of the Sept5 protein but cannot bind in the presence of $\alpha$-SNAP. Binding of Sept5 and syntaxin takes place at the H3 domain of syntaxin which is the same motif where both SNAP-25 and VAMP bind to form the docking fusion complex (Beites et al., 2005). The above makes Sept5 an intriguing candidate protein for comprising a portion of those filaments visualized between SVs and AZs, and may be involved in the determination of VGCC-SV coupling insofar as they may spatially separate SVs from the AZ membrane where VGCCs are located.

Here we investigate whether changes in the degree of Ca$^{2+}$-domain overlap (Ca$^{2+}$-domain cooperativity, $m$) produces measurable alterations in the timing of SV release. By measuring SD, in response to identical AP-input waveforms, from immature (high-$m$) and mature (low-$m$) synapses, we were able to isolate changes in release kinetics to factors downstream of Ca$^{2+}$ influx. In addition, using the same presynaptic voltage commands employed to determine $m$, we demonstrated that the dependence of SD on $I_{\text{Ca}}$ is influenced strongly by the spatial arrangement of VGCCs and SVs. Next,
using synapses from immature Sept5−/− mice, we found that cooperativity (m) was reduced as compared to Sept5+/+ synapses suggesting a role for the Sept5 protein in determining SV localization. This was confirmed in transmission electron microscopy (tEM) images in which the number of docked SVs was increased and the average separation distance between SV and AZs was significantly reduced in immature Sept5−/− synapses. Finally, we found that Sept5 was not directly involved in the refilling or transiting of SVs between pools, but may play some role in determining the number of available quanta in the RRP, during periods of intense activity, and in mature synapses.

From these experiments, we conclude that Sept5 is involved in establishing the increased spatial separation between SVs and AZs, and therefore VGCCs and SVs, observed in immature synapses, a role that either changes or is immeasurable in mature synapses. Furthermore, different spatial arrangements of VGCCs and SVs, besides altering the input-output relationship of the synapse, also serve to determine the range of release kinetics occurring in response to any range of I_{Ca} input.
5.2 RESULTS

5.2.1 Developmental Shortening in Synaptic Delay Occurs Downstream of Ca\(^{2+}\)-Influx.

Investigating changes in the timing of synaptic events during development requires precise separation of the pre- and postsynaptic elements of neurotransmission (Fedchyshyn & Wang, 2007). Further complicating the interpretation of such data are the numerous developmental adaptations that take place at the calyx of Held, resulting in fundamental alterations to the timing and robustness of SV release. In particular, developmental changes in the timing, shape, and size of presynaptic APs can profoundly affect the magnitude and kinetics of Ca\(^{2+}\) influx into the presynaptic terminal (Taschenberger & von Gersdorff, 2000; Lin & Faber, 2002; Taschenberger et al., 2002; Bollmann & Sakmann, 2005). Since SV release depends strongly on the nature of the Ca\(^{2+}\) signal, careful control of synaptic input is crucial for delineation of developmental changes that occur downstream.

To investigate whether developmental tightening of the coupling between VGCCs and SVs alters the timing of transmitter release, we recorded APs from a P10 current-clamped calyx, in response to afferent stimulation (110mV amplitude, 0.6ms half-width), and used this waveform to as a presynaptic voltage-command (Pre V-Com) to establish equivalent synaptic input in both immature (P8-12) and mature (P16-18) synapses. Applying this voltage paradigm to either immature or mature presynaptic terminals evoked calcium currents (I\(_{Ca}\)), and the resulting excitatory postsynaptic currents (I\(_{EPSC}\)), from pairs of simultaneously voltage-clamped calyx of Held-MNTB neurons (Fig. 5.1A). In 1mM [Ca\(^{2+}\)]\(_o\), both immature and mature calyces produced I\(_{Ca}\) of similar amplitude, in response to the same input AP (AP\(_I\)), however, I\(_{EPSC}\) was larger in mature synapses than in immature ones consistent with previous reports (Fedchyshyn & Wang, 2005; Yang & Wang, 2006). When records from immature and mature synapses were aligned by their I\(_{Ca}\) peak, we observed that mature synapses had a significantly shorter synaptic delay (SD) than did immature synapses given the same input waveform (0.09±0.02ms vs. 0.34±0.02ms, p<0.05, n=6/6) (Fig. 5.1A,B).

When [Ca\(^{2+}\)]\(_o\) was elevated to 2mM, immature synapses showed a significant decrease in their SD as compared with the same group in 1mM [Ca\(^{2+}\)]\(_o\) (0.23±0.03ms, p<0.05, n=5/6), suggesting that altering the magnitude of I\(_{Ca}\) by altering the driving force for Ca\(^{2+}\), can affect SD independent of the input waveform (Borst & Sakmann, 1999; reviewed in Lin & Faber, 2002; Gentile & Stanley, 2005). Furthermore, the responsiveness of the release machinery to a given Ca\(^{2+}\) input appears to differ between immature and mature synapses, becoming more rapid with maturity.

In response to these data, we investigated the possibility that developmental changes in VGCC-SV spatial coupling could account for the observed differences in SD.

5.2.2 The Relationship Between Synaptic Delay & Ca\(^{2+}\)-influx Depends on [Ca\(^{2+}\)]\(_o\) & Development.

In the immature calyx of Held-MNTB synapse, many VGCCs contribute Ca\(^{2+}\) ions to trigger the release of a single SV (“microdomain” modality), with the need for this cooperative action of VGCCs decreasing with development (“nanodomain” modality) (Borst & Sakmann, 1999; Wu et al.,1999;
Figure 5.1: Changes in Synaptic Delay With Development & Altered [Ca$^{2+}$].

(A) Paired presynaptic $I_{\text{Ca}}$ (middle panel) and postsynaptic $I_{\text{EPSC}}$ (bottom panel) from an immature (black, P10) and mature (grey, P18) synapse in response to the AP waveform command shown (top panel) aligned by $I_{\text{Ca}}$ peak. Solid vertical line shows the time of $I_{\text{Ca}}$ peak while dashed lines show the time of $I_{\text{EPSC}}$ onset for immature (black) and mature (grey) synapses. (B) Pooled data showing synaptic delay vs. age and [Ca$^{2+}$]. Asterisks denote significance (p<0.05, Student’s t-test) against the immature group in 1mM [Ca$^{2+}$]$_o$ (center bar).
Bollmann et al., 2000; Meinrenken et al., 2002; Fedchyshyn & Wang 2005). Besides the number of VGCCs involved, generally represented as the exponent of a synapses input/output transfer function (cooperativity, $m$ from $I_{\text{EPSC}} \propto [I_{\text{Ca}}]^m$), the magnitude of $I_{\text{Ca}}$ through any given channel also, in part, determines the number of VGCCs required to satisfy the Ca$^{2+}$ binding criterion of the Ca$^{2+}$ sensor (i.e. synaptotagmin) (Gentile & Stanley, 2005; Fedchyshyn & Wang, 2005).

To determine how the number of activated VGCCs in either release modality affects SD, we applied voltage steps with constant amplitudes and rise/decay phases, but increasing plateau durations, as voltage-commands to both immature and mature voltage-camped calyces (-80 to +60mV, 100µs increments in plateau time) (Fig. 5.2A, top panel). These voltage steps produced increasing presynaptic $I_{\text{Ca}}$ and postsynaptic $I_{\text{EPSC}}$ from synapses of both developmental stages by sequentially recruiting increasing numbers of VGCCs into the cumulative $I_{\text{Ca}}$ (Fig. 5.2A, middle & bottom panel).

When we plotted the normalized $I_{\text{Ca}}$ current density against normalized SD we found a relationship, well-described by a linear equation, the slope of which we termed “slope factor” ($s$), and representing the dependence of SD on the number of VGCCs contributing to $I_{\text{Ca}}$ (Fig. 5.2B).

When $s$ was calculated for immature and mature synapses in 1mM [Ca$^{2+}$]$_o$, we found that immature synapses had a significantly greater/steeper $s$ than did mature synapses ($1.4 \pm 0.1$ vs. $0.7 \pm 0.2$, n=14/7) (Fig. 5.2C). This suggests that rate at which SVs are recruited for release, downstream of Ca$^{2+}$ influx, is more steeply dependent on the number of VGCCs contributing to $I_{\text{Ca}}$ in immature terminals than in mature ones. Given the parallel developmental dependency of synaptic output ($I_{\text{EPSC}}$) on $I_{\text{Ca}}$ (cooperativity, $m$), we hypothesized that the same developmental differences in the underlying subsynaptic arrangement of VGCCs and SVs that determines $m$ may also affect $s$. That is, the loose coupling of VGCCs and SVs in immature terminals requires the cooperative action of many VGCCs to release a SV, the release probability ($P_r$) and kinetics of which depend largely on the number of VGCCs recruited. In contrast, the tight coupling in mature synapses, and the need for few VGCCs to trigger SV release, results in a $P_r$ that depends largely on the VGCC open-probability, for a given AP waveform, rather than on whether the cooperative contribution of many Ca$^{2+}$ microdomains occurs. Thus, if $P_r$, at an individual release site, determines the kinetics of SV release and is relatively resistant to changes in the number of VGCCs recruited, then the kinetics of SV release should show a parallel resistance as $I_{\text{Ca}}$ increases. In fact, we observe a shallow dependence of SD on $I_{\text{Ca}}$ ($s$) in mature synapses, in correlation with a comparatively weak dependence of $I_{\text{EPSC}}$ on $I_{\text{Ca}}$ ($m$), supporting the idea that these dependencies may be linked through $P_r$.

A prediction that can be made from this hypothesis is that experimental manipulation affecting $m$ should have parallel effects on $s$. Increasing the size of the Ca$^{2+}$ microdomains, by increasing the driving force for Ca$^{2+}$, reduces $m$ to a larger extent in immature synapses than in mature ones most likely by decreasing the requirement for cooperative interaction of VGCCs (Fedchyshyn & Wang, 2005). If $m$ and $s$ are similarly affected by changes in $P_r$, then increasing the driving force for Ca$^{2+}$ should also reduce $s$. To test this prediction, we repeated measurements of both $m$ and $s$ in 2 and 3mM [Ca$^{2+}$]$_o$, in immature synapses, and in 0.5 and 2mM [Ca$^{2+}$]$_o$, in mature synapses. When experiments were conducted below 1mM [Ca$^{2+}$]$_o$, in immature synapses, insufficient SV release was triggered to accurately measure either $m$
Figure 5.2: Dependence of Slope Factor & Cooperativity on $[\text{Ca}^{2+}]_o$ & Development.

(A) Paired presynaptic $I_{\text{ca}}$ and postsynaptic $I_{\text{EPSC}}$ recordings from an immature (middle panel, P8-12) and mature (bottom panel, P16-18) synapse in response to the presynaptic voltage-command waveform shown (top panel). Boxed regions are magnified 3x in insets. (B) Plot of normalized synaptic delay vs. normalized $I_{\text{ca}}$ current density for the example traces shown in A. Solid lines are least-squares regression fits through the data shown used to determine the slope (slope factor, $s$).

(C) Summary data showing slope factor ($s$) for various $[\text{Ca}^{2+}]_o$ in immature and mature synapses. (D) Summary data showing $\text{Ca}^{2+}$ domain cooperativity ($m$) for various $[\text{Ca}^{2+}]_o$ in immature and mature synapses.
or s. In contrast, when experiments were performed at 3mM Ca$^{2+}$, mature synapses, $I_{Ca}$ and $I_{EPSC}$ saturated too rapidly, upon broadening of the AP-waveform, to acquire measurements of m or s that did not potentially contain elements of either $I_{Ca}$ or $I_{EPSC}$ saturation (Fig. 5.2A). As a result, the tested range of [Ca$^{2+}$], for each age group was confined to those shown (Fig. 5.2C,D). In immature synapses, elevating [Ca$^{2+}$]$_o$ significantly lowered m from 5.5±0.5 to 3.5±0.5 and 2.6±0.4 for 1, 2, and 3mM [Ca$^{2+}$]$_o$, respectively (n=14/6/5). In mature synapses, lowering [Ca$^{2+}$]$_o$ from 1 to 0.5mM significantly increased m from 3.0±0.4 to 5.1±0.3 (n=7/5) while increasing [Ca$^{2+}$]$_o$ to 2mM significantly lowered m to 2.1±0.2 (n=6) (Fig. 5.2D). In either of the [Ca$^{2+}$]$_o$, tested between age groups (1 and 2mM), immature synapses showed significantly higher m values than in mature ones as expected from previous reports (Gentile & Stanley, 2005; Fedchyshyn & Wang, 2005).

When s was calculated from the data above it was found to parallel the trends observed in $m$. Immature synapses had s values of 1.4±0.1, 0.77±0.09, and 0.6±0.1 for 1, 2, and 3mM [Ca$^{2+}$]$_o$ respectively (n=14/6/5) while mature synapses has s values of 1.3±0.2, 0.7±0.2, and 0.7±0.1 for 0.5, 1, and 2mM [Ca$^{2+}$]$_o$ respectively (n=5/7/6) (Fig. 5.2C). Mature synapses showed significantly lower s values than immature synapses for 1mM [Ca$^{2+}$]$_o$, but not in 2mM [Ca$^{2+}$]$_o$ (p<0.05, Student's t-test). This confirms that by raising or lowering [Ca$^{2+}$]$_o$, the need for VGCC overlap can be decreased or increased altering the dependency of both $I_{EPSC}$ and SD on $I_{Ca}$ in a parallel manner. However, further increases in [Ca$^{2+}$], above 3mM, may not further reduce the requirement for VGCC domain overlap as a large Ca$^{2+}$ domain, through a single VGCC, may be sufficient for release of a single SV, thus establishing a lower limit for both m and s (Stanley, 1993; Gentile & Stanley, 2005).

5.2.3 Shortening of Synaptic Delay Depends on the Subtype & Localization of Voltage-Gated Calcium Channels.

To further explore the role of VGCC-SV coupling in determining s, we took advantage of the fact that the VGCC subtypes mediating SV release at the calyx of Held changes during a short window of postnatal development. Prior to ∼P11, release is mediated by an equally-effective mix of N and P/Q-type VGCCs with similarly loose coupling to SVs. At approximately P10/11, N-type VGCCs begin to move away from the AZs mediating a decreasing portion of release until they nearly completely depart the AZs after P12. During the same period, P/Q-type VGCCs couple more tightly to SVs and mediate the overwhelming majority of release (Iwasaki et al., 2000; Iwasaki & Takahashi, 1998; Wu et al., 1999; Fedchyshyn & Wang, 2005). By adding VGCC subtype-specific toxins we isolated the contribution of different VGCCs, with distinct spatial coupling arrangements to SVs, and investigated how this affected $s$. Application of the P/Q-type specific toxin ω-agatoxin-IVA (100nM) in 1mM [Ca$^{2+}$]$_o$ did not significantly effect s (1.1±0.1 vs. 1.4±0.1, n=8/14) compared to 1mM [Ca$^{2+}$]$_o$ alone, in agreement with unchanged m values under the same conditions (Fig. 5.2D, 5.3A,B,C). To assay the role of P/Q-type VGCCs in determining s, immature synapses were subdivided into two groups, P8/9 and P11/12. The P8/9 group employs P/Q-type VGCCs which have a similar coupling efficacy to N-type VGCCs. The P11/12 group
Figure 5.3: Dependence of Slope Factor on the Ca²⁺ Channel Subtype Mediating Release in Immature Synapses.

(A) Paired presynaptic I_{Ca} and postsynaptic I_{EPSC} recordings, in response to the voltage-command protocol shown (top panel), from immature synapses. Recordings were performed in the presence of 100nM ω-atx (top panel) and 1μM ω-ctx from which a younger (middle panel) and older (bottom panel) subgroup were created. Boxed regions are magnified 1.6x in insets. (B) Plot of normalized synaptic delay vs. normalized I_{Ca} current density for the example traces shown in A. Dashed lines are least-squares regression fits through data shown. (C) Summary data showing slope factor (s) for the conditions shown in A. (D) Summary data showing slope factor (s) with and without presynaptic loading of 10mM EGTA in immature synapses. Asterisks denote significance (Student’s t-test, p<0.05) within or across developmental stage as noted for all figures.
employs P/Q-type VGCCs that are coupled more tightly to the SVs (Fedchyshyn & Wang, 2005). Addition of ω-conotoxin-GVIA (1μM) in 1mM [Ca^{2+}]_o to the P8/9 subgroup resulted in similar s values to those in the absence of the toxin (p>0.05, Student’s t-test). However, in the P11/12 subgroup, blocking of N-type VGCCs significantly lowered s as compared to the P8/9 subgroup (1.5±0.2 vs. 0.8±0.2, P8/9 vs. P11/12, n=8/7) (Fig. 5.3C). The observed changes in s, that occur when specific populations of VGCCs are blocked, parallel similar changes in m under the same conditions, lending more evidence that both m and s are determined, at least in part, by the spatial coupling of VGCCs and SVs.

Although, immature synapses contain mostly loosely-coupled VGCC-SV units (using the microdomain release modality), they also contain a smaller population of tightly-coupled units (using the nanodomain release modality), as confirmed by the fact that presynaptic loading of 10mM EGTA does not completely attenuate transmitter release (Borst & Sakmann, 1996; Fedchyshyn & Wang, 2005). During development, the distribution of loosely versus tightly-coupled units in the synapse shifts to contain mostly tightly-coupled nanodomain VGCC-SV units. This transitional period provides another means by which to test the role of VGCC-SV coupling in determining s. Addition of EGTA to the presynaptic terminal functionally excludes loosely-coupled units from contributing to SV release by buffering Ca^{2+} ions in transit between VGCCs and distant neighbouring SVs (Adler et al., 1991, Borst et al., 1995; Borst & Sakmann, 1996; Naraghi & Neher, 1997; Fedchyshyn & Wang, 2005). Thus presynaptic loading of EGTA allows only those Ca^{2+} ions originating from a nearby VGCC to contribute to release of SVs. Under these conditions we expect that the measured s would be reminiscent of those observed in other low-m conditions (i.e. mature synapses or high [Ca^{2+}]_o). When 10mM EGTA was loaded into immature terminals, s decreased significantly from 1.4±0.1 to 0.9±0.2 (p<0.05, Student’s t-test, n=14/7) consistent with s-values observed in both elevated [Ca^{2+}]_o in the presence of ω-conotoxin-GVIA (P11-12), and in mature synapses at 1mM [Ca^{2+}]_o (Fig. 5.2D, 5.3C,D).

Together these data suggest that the dependence of SD on I_{Ca} (s) is determined specifically by the same factors that determine m, being the spatial localization of VGCCs relative to SVs.

5.2.4 A Greater Number of Synaptic Vesicles Are Docked in Mature Synapses Than in Immature Ones.

Having evidence suggesting that VGCC-SV localization tightens during development, we asked whether, at a morphological and structural level, immature and mature AZs differ in the arrangement of their SVs. To address this issue we performed transmission electron microscopy (tEM) on brainstem slices containing the MNTB. Upon initial inspection of calyceal AZs from the resulting images, we found that a greater number of SVs appeared to be in physical contact with the AZ membrane in mature synapses than in mature ones (Fig. 5.4A). To quantify this observation, we measured separation distances between SVs and AZs, in immature and mature synapses, and constructed normalized separation distance histograms for both age groups (see section 2.6.3). In mature synapses, 23% of SVs were found to be docked (<10nm) to the AZ compared to only 14% in immature synapses (SVs=1678/1282 vesicles, n=6/6) (Fig. 5.4B). Immature synapses also had a slightly larger proportion of
Figure 5.4: Relative Localization of Synaptic Vesicles to Active Zones in Immature & Mature Calyces.

(A) TEM images, taken from a P12 (left panel) and P17 (right panel) synapse, of visually identified active zones. Synaptic vesicles closest to active zones have been shaded in red for clarity with AZ bounds denoted by red dashed lines. Each image in each panel recorded from a distinct calyx. (B) Summary relative histogram plotting vesicle separation distance from active zones for immature (black) and mature (red) synapses. Dashed vertical line marks the arbitrary point at which all vesicles to the left are considered morphologically “docked” (10nm). (C) Schematic diagram of proposed model of Ca$^{2+}$ channel-synaptic vesicle coupling modality incorporating developmental synaptic vesicle separation difference from the active zones. The microdomain (left panel) and nanodomain (right panel) release modalities refer to those proposed in Fedchyshyn & Wang, 2005.
SVs separated by between one and two SV diameter(s) compared with mature synapses (54% vs. 47%) with the overall distributions being significantly different (K-S test, P<0.05) (Fig. 9.2.5C).

Given these results we refined our model, previously proposed in Fedchyshyn & Wang (2005), describing a developmental tightening in the coupling of VGCCs and SVs, to include a larger average SV-AZ separation distance in immature synapses as compared to mature ones (Fig. 5.4C). We hypothesize that this increased separation distance underlies the larger requirement for VGCC cooperation in immature synapses and the steep dependence of both \( m \) and \( s \) at this developmental stage.

5.2.5 Septin 5 Is Found in the Presynaptic Terminal of Immature Synapses.

In light of our observation that fewer SVs in the immature terminal are docked at AZs, we sought to examine by what means the SV-AZ separation distance was established. One member of the Septin family of proteins, Septin 5 (Sept5), provided an attractive molecular candidate for this role. Sept5, or CDCrel-1, is a member of the Septin family of proteins originally identified as being involved in cell division, in particular, in membrane fission (Hartwell, 1971; Byers et al., 1976; Longtine et al., 1996; McKie et al., 1997; Zeiger et al., 1997). More recently, Sept5 was identified as binding to the SNARE protein syntaxin1A/2 as well as a number of other members of the Septin proteins including Sept2, and Sept7 (Peng et al., 2002). Furthermore, over-expression of Sept5 was found to inhibit SNARE-mediated vesicular release of human growth hormone from HIT-T15 and potentiate release when a dominant-negative construct of Sept5 was over-expressed in the same cell lines (Beites et al., 1999). Sept5 binds to the 7S complex but is displaced by \( \alpha \)-SNAP, a process which is hypothesized to allow for formation of the trans-SNARE complex and subsequent docking of SVs (Beites et al., 2005). Two mechanisms for the action of Septin proteins in mediating this role have been suggested; (i) Septin filaments (containing Sept5) bind to 7S complexes on the SV and the AZ preventing docking of the SV at the AZ, (ii) Sept5 containing filaments bind across the AZ to 7S complexes and prevent docking of SVs (Fig. 5.5A) (adapted from Beites et al., 2005). In fact, SVs refilling the RRP have been shown to come to rest 20nm from AZs in goldfish retinal bipolar neurons, while filament-like strands have been localized in this space. Similar filaments have been observed linking SVs to each other in rat/mouse brain and frog neuromuscular junction, all locations in which Sept5 has been localized (Hirokawa et al., 1989; Kinoshita et al., 2000, Zenisek et al., 2000). Interestingly, rat brain-associated Septin filaments have also been shown to form lengths in multiples of 25nm (Hsu et al., 1998), in line with separation distances measured in the goldfish. Taken together, the above observations suggest a strong candidacy for Sept5 in defining SV docking and localization.

To ensure that Sept5 was native to the Calyx of Held, we used antibody conjugated fluorophores to label Sept5 protein, a presynaptic marker (VAMP), and DNA in brainstem slices taken from Sept5\(^{+/+}\) and Sept5\(^{-/-}\) mice (see section 2.6.4). Sept5 expression was observed in the majority of VAMP labelled presynaptic neurons while relatively little expression was observed in MNTB neurons (Fig. 5.5B, top panel). Magnification of a single synapse (Fig. 5.5B, bottom panel) revealed that Sept5 is expressed...
Figure 5.5: Models for Septin Interaction & Localization of Sept5 at the Presynaptic Nerve Terminal.

(A) Schematic diagram of potential mechanisms of Septin proteins at the presynaptic nerve terminal. Septin filaments (containing Sept5) may act to tether synaptic vesicles to active zones by binding to 7S complexes on both (left panel). Alternatively, Septin filaments may bind to 7S complexes across active zones restricting access to vSNAREs (right panel). (B) Calyx of Held-MNTB synapses, from P12 Sept5+/− (left panel) and Sept5−/− (right panel), stained for Sept5 (green), VAMP (red), and DNA (blue). Digital magnification of a single synapse from each genotype is shown (bottom panels).
presynaptically and co-localizes with VAMP. Loss of Sept5 protein was confirmed in slices prepared from age-matched Sept5−/− mice (Fig. 5.5B, right panels). Mature Sept5+/+ and Sept5−/− synapses were also labelled as above; however, at this developmental stage, images showed high background staining for Sept5 (data not shown). This is most likely due to the technical difficulties imposed by using a mouse antibody (Sept5) on mouse tissue and the increased density of tissue at this developmental stage. From these images it appears that Sept5 is expressed in the mature calyx of Held; however, inference into relative expression level or fine localization of the protein cannot be made at this point. Despite these difficulties in mature synapses, these results confirm the presence and absence of Sept5 protein in the immature calyx of Held of Sept5+/+ and Sept5−/− synapses respectively.


The role of Sept5 has been previously tested in hippocampal synapses (Peng et al., 2002). In this study, little difference was observed between Sept5+/+ and Sept5−/− synapses in terms of paired-pulse facilitation (PPF), post-tetanic potentiation (PTP), synaptic depression, or long-term potentiation (LTP). To confirm this finding we assayed synaptic transmission in both immature and mature Sept5+/+ and Sept5−/− mice at the calyx of Held-MNTB synapse. Afferent evoked IEPSC from immature synapses were found to be significantly larger in Sept5−/− than in Sept5+/+ synapses while no significant difference was observed in mature synapses (7.6±0.3 vs. 6.4±0.3nA and 13.7±0.8 vs. 12.2±0.7nA; immature and mature respectively; p<0.05) (Fig. 5.6A,B). If the loss of Sept5 potentiates vesicular release, as found in HIT cells (Beites et al., 1999), then an increased in evoked IEPSC would be in-line with the current proposed model of Sept5 interaction at the AZ (Beites et al., 2005). However, it is possible that an increase in AP width may occur in Sept5−/− calyces and underlie the observed increase in IEPSC. To address this issue, we performed simultaneous whole-cell pre- and postsynaptic recordings from the calyx of Held and MNTB neurons of immature and mature Sept5+/+ and Sept5−/− mice. We used an AP waveform, afferent-evoked in an immature (P10) calyx, as a voltage-command to generate I_Ca and the corresponding IEPSC from these synapses in 1mM [Ca²⁺]o (see section 2.2.5). This allowed for determination of Pᵣ independent of potential changes in AP waveform. Using this paradigm, the increased IEPSC observed in immature Sept5−/− synapses, in response to afferent-evoked APs, was again observed. In mature synapses, no difference in IEPSC amplitude was detectable consistent with data gained from afferent evoked stimulation (3.2±0.2 vs. 2.0±0.3 and 15.9±0.6+14.9±0.7nA, immature and mature respectively) (Fig. 5.6C,E). In addition, a decrease in SD was observed in immature Sept5−/− synapses, conducive with an increase in Pᵣ over Sept5+/+ synapses of similar developmental stage (0.3±0.02 vs. 0.38±0.02ms, P<0.05) (Fig. 5.6C,D). In mature synapses, differences in SD and IEPSC, using the APᵣ presynaptic voltage-command, may have been masked due to the unusually wide AP used to generate sufficient IEPSC from immature synapses (0.16±0.01 vs. 0.17±0.01ms, P>0.05) (Fedchyshyn & Wang, 2005). Indeed, IEPSC values in mature synapses were much larger than those typically observed at 1mM [Ca²⁺]o in response to evoked APs (Joshi & Wang, 2002). However, at least in the immature Sept5−/− synapse, Pᵣ appears to be elevated.
Figure 5.6: Synaptic Transmission at Sept5<sup>−/−</sup> & Sept5<sup>+</sup> Synapses.

(A) Evoked post synaptic I<sub> EPSC </sub> from immature (left panel) and mature (right panel) Sept5<sup>−/−</sup> (black) and Sept5<sup>+</sup> (red) synapses.  (B) Summary data showing average I<sub> EPSC </sub> amplitude for the experimental conditions shown in A.  (C) Presynaptic voltage-command AP (top panels) with resultant presynaptic I<sub> Ca </sub> (middle panels) and postsynaptic I<sub> EPSC </sub> (bottom panels) for immature (left panels) and mature (right panels) Sept5<sup>−/−</sup> (black) and Sept5<sup>+</sup> (red) synapses.  (D) Summary data of synaptic delay for the conditions shown in C.  (E) Summary data for I<sub> EPSC </sub> amplitude in response to the AP, presynaptic voltage-command and conditions shown in C.  (F) Cumulative RRP current density as a function of event number for immature (left panel) and mature (right panel) Sept5<sup>−/−</sup> (black) and Sept5<sup>+</sup> (red) synapses.  Solid lines represent least-squares linear fit to region of highest R<sup>2</sup>-value.  Dashed lines represent upper and lower 95% confidence limits.  Asterisks represent significance to P<0.05 within or between experimental groups as noted (Student’s t-test).
In addition to potential differences in release properties in $Sept5^{+/}$ synapses, we asked whether the loss of $Sept5$ may alter the size of the RRP. Loss of $Sept5$ resulted in an increase in the RRP current density, calculated as described in Schneggenburger et al. (1999), in both immature and mature synapses (Fig. 5.6F). Briefly, immature synapses were given trains of evoked APs (100Hz, 200ms) which generated depleting trains of $I_{EPSC}$. The current densities from these trains were summated cumulatively to generate plots (Fig. 5.6F), which were back-extrapolated to give the RRP current densities for both $Sept5^{+/}$ and $Sept5^{-/-}$ synapses. A similar protocol was used in mature synapses however trains of 200Hz for 100ms were used. Immature $Sept5^{-/-}$ and $Sept5^{-/-}$ synapses had RRP current densities of 16.3±0.3 and 22.6±0.1nA•ms respectively, while mature $Sept5^{+/}$ and $Sept5^{-/-}$ synapses had RRP current densities of 21.4±0.3 and 31±1nA•ms respectively. These data suggest that at the calyx of Held, loss of $Sept5$ results in an increase in $P_{n}$ in immature synapses, and an increased availability of SVs during high-frequency activity. Based on these results we sought to determine if alterations in VGCC-SV coupling could underlie the increased $P_{n}$ observed in immature $Sept5^{-/-}$ synapses.

5.2.7 Loss of $Sept5$ Reduces $Ca^{2+}$ Channel Domain Cooperativity & the Dependence of Synaptic Delay on $I_{Ca}$.

To further investigate the effect of a loss of $Sept5$, we measured cooperativity ($m$) in $Sept5^{+/}$ and $Sept5^{-/-}$ synapses from both immature and mature mice. If $Sept5$ acts to prevent tight localization or docking of SVs with AZs, then loss of the protein should result in a tighter coupling and a reduced requirement for the cooperative action of VGCCs in releasing SVs. Using the presynaptic voltage-command protocol shown in Fig. 5.2A, we generated presynaptic $I_{Ca}$ and postsynaptic $I_{EPSC}$ form $Sept5^{+/}$ and $Sept5^{-/-}$ synapses (Fig. 5.7A). When we compared $m$ values between immature $Sept5^{+/}$ and $Sept5^{-/-}$ synapses we found that $Sept5^{-/-}$ synapses had a significantly lower $m$ value than that of $Sept5^{+/}$ synapses (5.0±0.4 vs. 3.3±0.1, $p<0.05$, n=9/7) (Fig. 5.7B). We repeated these experiments in mature $Sept5^{+/}$ and $Sept5^{-/-}$ synapses and found no significant difference between their respective $m$-values (2.7±0.1 vs. 3.0±0.2, $P>0.05$, n=6/6) (Fig. 5.7D). These data suggest that loss of $Sept5$ decreases the requirement for $Ca^{2+}$-domain overlap in releasing SVs from immature synapses, a role which either differs or is immeasurable in the mature synapse.

As above, we predicted that slope factor ($s$) should follow those trends observed in $m$. When we measured $s$ from immature $Sept5^{+/}$ and $Sept5^{-/-}$ synapses we found that $s$ was also decreased when $Sept5$ protein was absent (1.4±0.2 vs. 0.8±0.1, $P<0.05$, n=9/7) (Fig. 5.7A,C). As with $m$, no significant difference in $s$ was observed between mature $Sept5^{+/}$ and $Sept5^{-/-}$ synapses (0.8±0.2 vs. 0.6±0.1, $P>0.05$, n=6/6) (Fig. 5.7E). These data lend further evidence to suggest that, when alterations to the spatial coupling of VGCCs and SVs or the requirement of $Ca^{2+}$-domain overlap occur, parallel effects on both $m$ and $s$ are observed. Furthermore, these data implicate $Sept5$ as being a key protein modulating $m$ and $s$ in the immature synapses, most likely through a common effect on VGCC-SV coupling.
Figure 5.7: Release Modality & Slope Factor in Sept5°° & Sept5° Synapses.
(A) Paired presynaptic $I_{Ca}$ and postsynaptic $I_{EPSC}$ recordings, in response to the voltage-command protocol shown in Fig. 5.3A, from immature Sept5°° (left panel) and Sept5° (right panel) synapses. Boxed regions are magnified 1.6x in insets. (B) Log-log plot of normalized $I_{Ca}$ vs. $I_{EPSC}$ for the recordings in A. Dashed lines are least-squares regression fits through the data shown. (C) Plot of normalized synaptic delay vs. normalized $I_{Ca}$ current density for the example traces shown in A. (D) Summary data showing $m$ values for immature and mature Sept5°° and Sept5° synapses. (E) Summary data showing $s$ values for immature and mature Sept5°° and Sept5° synapses. Asterisks represent significance (p<0.05, Student’s t-test) within or across developmental stage as indicated.
Figure 5.8: Morphological Localization of Synaptic Vesicles in Sept5\(^{+/-}\) & Sept5\(^{-/-}\) Synapses.
(A) TEM images, taken from immature Sept5\(^{+/-}\) and Sept5\(^{-/-}\) (P9, left panel) and mature Sept5\(^{+/-}\) and Sept5\(^{-/-}\) (P10, right panel) synapses, of visually identified active zones. Synaptic vesicles closest to active zones have been shaded in red for clarity with AZ bounds denoted by red dashed lines. Each image in each panel recorded from a distinct calyx. (B) Summary normalized histogram plotting vesicle separation distance from active zones for immature Sept5\(^{+/-}\) (black), immature Sept5\(^{-/-}\) (red), mature Sept5\(^{+/-}\) (dashed black), and mature Sept5\(^{-/-}\) (dashed red) synapses. (C) mEPSC recordings (30s) from immature Sept5\(^{+/-}\), immature Sept5\(^{-/-}\), mature Sept5\(^{-/-}\), and mature Sept5\(^{-/-}\) postsynaptic MNTB neurons. (D) Summary data showing mEPSC frequency for immature and mature Sept5\(^{+/-}\) and Sept5\(^{-/-}\) synapses. (E) Summary data showing mEPSC amplitude for immature and mature Sept5\(^{+/-}\) and Sept5\(^{-/-}\) synapses.
5.2.8 Immature Sept5⁺/+ Synapses Have Fewer Morphologically Docked Vesicles Than Do Sept5⁻/⁻ Synapses.

Having evidence that loss of Sept5 reduced the need for cooperative interaction of Ca²⁺ domains in immature synapses, we sought confirmation that the reduction in m was due to a decrease in the spatial separation of SVs and AZs in immature Sept5⁻/⁻ synapses. To assay this further we performed tEM of sections, prepared from immature and mature Sept5⁺/+ and Sept5⁻/⁻ synapses (Fig. 5.8A), and measured the separation distance between SVs and AZs. We found a significant increase in the proportion of docked SVs in immature Sept5⁻/⁻ synapses as compared to the age-matched Sept5⁻/⁻ controls (21% vs. 14%, SVs=1678/1492, n=6/6, p<0.05, Student’s t-test) Due to the difference in the number of docked SVs, the overall distributions of SVs were significantly different (p<0.05, Kolmogorov-Smirnov test, see appendix Fig. 9.2.5D), while the distribution of vesicles with separation distances greater than 20nm was similar (Fig. 5.8B). Distributions for mature Sept5⁺/+ and Sept5⁻/⁻ synapses were not found to be significantly different as expected from their similar m and s values (K-S test, P>0.05, n=1694/1737) (Fig. 5.8B).

Given that the loss of Sept5 impacted the localization of SVs, we recorded spontaneous mini EPSCs (mEPSCs) to determine whether the quantal properties of these synapses had also changed as a result of the deletion. When mEPSCs were recorded from immature and mature Sept5⁺/+ and Sept5⁻/⁻ synapses, a characteristic increase in mEPSC frequency and amplitude was observed with development (Taschenberger et al., 2005) (Fig. 5.7C,D,E). However, no significant difference was observed for either parameter between Sept5⁺/+ and Sept5⁻/⁻ synapses in either age group. mEPSC frequency was 0.9±0.1 vs. 0.7±0.1 events•sec⁻¹ (p>0.05, n=17/16) and 3.4±0.3 vs. 3.9±0.4 events•sec⁻¹ (p>0.05, n=7/7) for immature and mature Sept5⁺/+ and Sept5⁻/⁻ synapses respectively (Fig. 5.7D). mEPSC amplitudes were 46±1 vs. 46±2pA (p>0.05, n=17/16) and 58±2 vs. 64±4pA (P>0.05, K-S test, n=7/7) for immature and mature Sept5⁺/+ and Sept5⁻/⁻ synapses respectively (Fig. 5.7E).

The lack of alteration to mEPSC amplitude or frequency, upon loss of Sept5, suggests that the Sept5 protein acts primarily in establishing Ca²⁺-dependent release of SVs and has little effect on spontaneous release. These data also exclude the possibility that changes in fundamental quantal properties, such as quantal size, account for the observed decrease in m and s in immature Sept5⁻/⁻ synapses.

5.2.9 Cooperativity & Slope Factor Correlate Across All Experimental Groups & Developmental Stages.

In light of the data acquired from Sept5⁺/+ and Sept5⁻/⁻ synapses, we revisited the hypothesis that the coupling of VGCCs and SVs, which determine m, also determine s. Pooling all of the data, collected from the calyx of Held-MNTB synapse under various conditions (Table 5.1), we plotted m vs. s (Fig. 5.8). We found that under all conditions in which Ca²⁺-domain overlap was required for the release of SVs (i.e. low [Ca²⁺]₀, immature synapses, etc…), the dependence of SD on I_{Ca} was always high. The converse was true when the requirement for Ca²⁺-domain overlap was reduced (i.e. high [Ca²⁺]₀, mature synapses,
Figure 5.9: The Dependence of Slope Factor on Cooperativity.  
(A) Correlation plot of cooperativity ($m$) vs. slope factor ($s$) for all experimental conditions tested. Solid line is a least-squares fit through the data shown. Data used to produce this figure are shown in Table 5.1.
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<td>6</td>
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<tr>
<td>1mM [Ca$^{2+}$], Sept5$^{+/}$</td>
<td>0.8±0.2</td>
<td>2.7±0.1</td>
<td>6</td>
</tr>
<tr>
<td>1mM [Ca$^{2+}$], Sept5$^{-}$</td>
<td>0.6±0.1</td>
<td>3.0±0.2</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5.1: Measurements of Slope Factor (s) & Cooperativity (m). Summary table containing the cooperativity (m) and slope factor (s) derived from the experimetal conditions and developmental stages listed. Data is derived from the figures of Chapter 5.
etc...). Furthermore, immature Sept5/ synapses, exhibiting uncharacteristically low m-values, similarly exhibited low s-values. Combined with the observation that immature Sept5/ synapses had a greater number of docked SVs than Sept5/+ synapses, this provides strong evidence that both m and s are influenced in a similar manner by the spatial intimacy of VGCCs and SVs.

A steep dependence between SD/I_EPSC and I_Ca, caused by loose coupling of VGCCs and SVs, increases the dynamic range of synapses in response to a given input range (I_Ca). Thus immature synapses are capable of much greater degrees of plasticity in the magnitude and timing of synaptic signals but also require significant Ca^{2+}-domain overlap, brought about through large I_Ca, to ensure high-fidelity synaptic transmission with minimal latency. In contrast, consistency in the timing and magnitude of synaptic transmission in mature synapses is ensured by decreasing the dynamic range of both I_Ca-I_EPSC and I_Ca-SD dependencies thereby decreasing the need for significant Ca^{2+}-domain overlap and the accompanying large I_Ca. These changes provide a means by which developing synapses can acquire their ability to reproduce high-frequency input signals, with high fidelity, while minimizing the site-specific Ca^{2+} influxes required for their initiation.
5.3 DISCUSSION

Having characterized a developmental change in release modality, in which VGCCs couple more closely to SVs with synaptic maturation (Fedchyshyn & Wang, 2005), we began to probe both the molecular mechanisms underlying this change and the impacts it has on the kinetics of synaptic transmission. We have taken advantage of our ability to perform high-quality simultaneous voltage-clamp recordings from pre and postsynaptic elements of the calyx of Held-MNTB synapse, and the distinct VGCC-SV coupling found at immature and mature developmental stages, to investigate how this adaptation contributes to the development and maintenance of high-fidelity neurotransmission at this synapse. Our results suggest that, independent of the input waveform and downstream of Ca\(^{2+}\) influx, the kinetics of SV release are much slower and have a broader dynamic range in immature synapses than in mature ones (Fig. 5.1). This implies that, once Ca\(^{2+}\) enters the terminal, its path in triggering a release event, or the actuation of the release event itself, is fundamentally different across development. A number of possible explanations exist for this difference;

If the Ca\(^{2+}\) sensor (i.e. synaptotagmin) were more sensitive to [Ca\(^{2+}\)], in mature synapses, the kinetics of Ca\(^{2+}\) binding and engagement of the release machinery could increase. Although the sensitivity of the mature Ca\(^{2+}\) sensor is not currently known, the high-sensitivity of the immature sensor (\(\sim 10\mu\text{M}\)) suggests that further sensitization may result in a decreased ability for individual SVs to distinguish true Ca\(^{2+}\) signals from noise (Bollmann et al., 2000; Schneggenburger & Neher, 2000). In addition, it has been shown that synaptotagmin 2 mediates Ca\(^{2+}\)-dependent SV release at both the immature and mature calyx of Held suggesting that, if a change in sensitivity occurs with development, it must arise from modifications other than a simple change in synaptotagmin subtype expression (Pang et al., 2006; Fox & Sanes, 2007; Xu et al., 2007). Future direct measurement of Ca\(^{2+}\) sensitivity in mature synapses, through flash-photolysis of caged Ca\(^{2+}\) compounds, will allow for resolution of this possibility.

Alternatively, developmentally distinct endogenous Ca\(^{2+}\) buffers, with distinct binding rates, may alter the effective spread of Ca\(^{2+}\) within the terminal and alter its effectiveness in triggering SV release. Due to the whole-cell recording configuration used in our experiments, any mobile endogenous buffer would be dialyzed-out by that contained within the recording electrode (0.5mM EGTA), providing equivalent buffer conditions across experimental groups. Recent evidence suggests that parvalbumin is the endogenous Ca\(^{2+}\) buffer at the immature calyx of Held and that it has similar binding rates but lower effective concentration to the EGTA used in our presynaptic electrodes (Müller et al., 2007). Also, fixed buffers have been shown to have little effect on the size and spread of Ca\(^{2+}\)-domains within the terminal, suggesting that, in our experimental conditions, changes in Ca\(^{2+}\) buffering cannot explain the observed developmental changes in SD (Meinrenken et al., 2002).

Finally, as proposed here, a change in the spatial separation of VGCCs and SVs, and the degree of VGCC cooperation required for generation of supra-threshold Ca\(^{2+}\) signals, could account for the developmental differences observed in SD. Both the kinetics of SV release, and the P, of individual SVs, are determined by the nature of the spatiotemporal [Ca\(^{2+}\)] profile experienced by the Ca\(^{2+}\) sensor. While P, depends only on the magnitude, SD depends on both the diffusion time and the magnitude of the
[Ca\(^{2+}\)] profile, both significantly altered by relative positioning of the SV and Ca\(^{2+}\) source(s) (Bollmann et al., 2000; Meinrenken et al., 2002; Meinrenken et al., 2003; Fedchyshyn & Wang, 2005). Thus, mature synapses should produce [Ca\(^{2+}\)] profiles that reach the SV faster and with larger peak amplitudes than do immature synapses, producing shorter synaptic delays in response to identical input waveforms. See appendix 9.3 for proof of principle of this hypothesis. Manipulation of [Ca\(^{2+}\)]\(_o\), while having only subtle effects on the rate of Ca\(^{2+}\) diffusion, can significantly increase the amplitude of the [Ca\(^{2+}\)] profile, this being especially relevant when long diffusion distances, such as in immature synapses, result in attenuated peak [Ca\(^{2+}\)] profile amplitudes (Llinás et al., 1981; Bollmann et al., 2002; Meinrenken et al., 2002).

Given that the microdomain coupling modality provides for a significantly larger range of synaptic output (I\(_{EPSC}\)) for a given range of I\(_{Ca}\), we asked whether SD would be similarly sensitive to ranges of Ca\(^{2+}\)-domain overlap (Fedchyshyn & Wang, 2005). We chose to modulate P\(_r\) by gradually increasing the number of recruited VGCCs, using successively wider AP waveforms, selectively increasing the chance that a sufficient summated Ca\(^{2+}\) signal would be generated in the vicinity of a SV and trigger its release. As the degree of Ca\(^{2+}\)-domain overlap, and thus the P\(_r\), increased, the kinetics of transmitter release quickened, producing an inverse linear relationship between I\(_{Ca}\) and SD (slope factor, s). This dependence was found to be significantly steeper in immature synapses than in mature ones. As the requirement for Ca\(^{2+}\)-domain overlap was decreased, by increasing [Ca\(^{2+}\)]\(_o\), both m and s decreased in parallel and to a greater extent in immature synapses. In contrast, decreasing [Ca\(^{2+}\)]\(_o\) in mature synapses increased both m and s-values to those expected of immature ones. Moreover, blocking of specific VGCC subtypes or presynaptic addition of EGTA to immature synapses, produced s-values that again correlated with the m-value typical of the spatial localization of that particular subtype of VGCC and the experimental conditions (Fig. 5.2, 5.3, 5.7, & 5.9).

Interestingly, many previous studies, in which SD was found to be invariant, may have been conducted under experimental conditions in which effective m-values were low (i.e. high [Ca\(^{2+}\)]\(_o\), tight VGCC-SV coupling, mature synapses) (Katz & Miledi, 1965; Barrett & Stevens, 1972; Llinás et al., 1981; Parnas et al., 1989). Our results raise the possibility that much of the debate regarding the variance/invariance of SD with changing P\(_r\) may be a consequence of the effect that VGCC-SV coupling and domain overlap has on the dynamic range of SD. In agreement with the data summarized by Gentile & Stanley (2005), regarding the Ca\(^{2+}\)-dependence of m from various experimental systems, our results show, in the same system and synapse, that both m and s depend similarly on the [Ca\(^{2+}\)]\(_o\), with this dependence being more dynamic in immature synapses. The relative insensitivity of SD to I\(_{Ca}\), observed in low-m conditions, most likely arises from the rapid and supra-threshold [Ca\(^{2+}\)] profile created in close proximity to an open and tightly-coupled VGCC. Moderate experimental adjustments made to the magnitude of I\(_{Ca}\) (i.e. increasing [Ca\(^{2+}\)]\(_o\)), or to whether additional VGCCs open in the vicinity (i.e. increased AP duration/width), are likely to have mild effects on the P\(_r\) and kinetics of a tightly-coupled SV. In contrast, spatiotemporal summation of a number of spatially heterogeneous VGCCs produces a wide range of [Ca\(^{2+}\)] profiles which, in turn, produce a wide range of SV release kinetics in immature synapses.
We next asked, given the myriad of presynaptic scaffolding proteins that have recently been identified and characterized, which one(s) may regulate the developmental change in VGCC coupling? At the frog neuromuscular junction (NMJ) and hair-cell ribbon synapse highly regular arrays of proteins and AZ structures are thought to be specialized for fast and reliable neurotransmission (Harlow et al., 2001; reviewed in Moser et al., 2006). Since the calyx of Held-MNTB synapse is also specialized for high-frequency, high-fidelity neurotransmission, it is reasonable to assume that proteins and structures with analogous roles in AZ organization should also exist at this synapse.

A change in VGCC-SV spatial coupling could occur at the AZ in three ways; first, the “lateral” separation may change through the developmental regulation of any number of proteins that link VGCCs to the release machinery (Catterall, 2000). Second, the relationship between VGCCs and the release machinery may remain constant while the “vertical” position of the SV changes due to developmental regulation of other proteins (Hsu et al., 1998; Zenisek et al., 2000). Finally, a combination of both mechanisms may underlie developmental tightening of the release modality. TEM images, taken from immature and mature AZs, show a lower number of docked SVs in immature synapses suggesting that a change in SV positioning, relative to the AZ, may indirectly underlie changes in VGCC-SV coupling (Fig. 5.4). A number of presynaptic proteins, located within the cytomatrix of the active zone (CAZ), have been implicated in SV stabilization/localization and may determine VGCC-SV localization including; (1) RIM which has been shown to interact with a number of other presynaptic proteins and is implicated in the priming and release step of SVs (Calakos et al., 2004). (2) ELKS/CAST which is through to properly localize RIM to the AZ (Ohtsuka et al., 2002). (3) Munc-13 which is crucial in SV acquiring a fusion competent state through its interaction with syntaxin (Augustin et al., 1999). (4) Piccolo/Bassoon, present only in vertebrates, can interact indirectly with both Rab3 and VAMP2 and is thought to play a role in retrieving endocytosed SVs from peri-AZ regions back to the AZ (Fenster et al., 2000). Indeed, mutations in the bassoon protein result in dissociation of ribbons from the presynaptic plasma membrane of retinal photoreceptors and cochlear hair cells (Dick et al., 2003; Khimich et al., 2005). (6) Finally Liprin-α interacts directly with RIM, ELKS, and CASK, indirectly with piccolo, and is implicated in the formation of AZ and the number of SVs associated with them (Zhen & Jin, 1999; Schoch et al., 2002; Kim et al., 2003).

Each of these proteins has been implicated in the assembly and arrangement of AZ components or the regulation of SV exocytosis and could regulate, directly or indirectly, the position of SVs in relation to the AZ membrane. However, here we have investigated the role of another protein, Septin 5 (Sept5), which has been shown previously to effect exocytosis through its interactions with the 7S SNARE complex (Beites et al., 1999, Beites et al., 2005).

Using synapses from Sept5−/− mice we first confirmed localization of the protein to the calyx of Held, and then investigate what impact, if any, loss of Sept5 would have on the localization of SVs throughout development. Septin proteins, including Sept5, form heteromeric filaments in length multiples of 25nm, which bind at both termini to syntaxin-1A while part of the 7S complex (Beites et al., 2005). Interestingly, SVs in the goldfish retinal bipolar come to rest approximately 20nm from the AZ membrane before becoming fusion competent (Hsu et al., 1998; Zenisek et al., 2000). In immature synapses, our
results suggest that Sept5, or Sept5 containing filaments, most likely do not establish this particular arrangement due to the lack of a secondary peak, around 20nm, in the SV-AZ distribution histogram from immature Sept5+/− synapses (Fig. 5.8B). A more likely scenario is the alternative arrangement proposed by Beites et al. (2005) in which Sept5 filaments lay across the AZ providing an “mesh” that prevents the docking step from taking place (Fig. 5.6A, right panel). This predicted role for Sept5 would increase VGCC-SV separation distances; however, not necessarily to the ~20nm distance observed in other systems (Hsu et al., 1998; Zenisek et al., 2000).

In the context of SV “docking”, Sept5 is thought to act as a brake for SV docking, that is, its action would be upstream to the process of SNARE binding. The process of SV “docking”, often referred to interchangeably as “priming” in the literature which we reserve for use in the context of the residual-Ca^{2+} hypothesis, likely involves a complex of RIMs and Munc13. In this complex, Rabs, attached to SVs in their GTP-bound state, likely interact with the AZ protein Munc13 through their mutual interaction with RIM (Dulubova et al., 2005). This tripartite complex is hypothesized to stabilize the SVs in a release-ready state. It is likely that the removal of Sept5, either via competition by α-SNAP or when deleted genetically, promotes or allows the formation of the Rab3A/α-RIM/Munc13-1 complex at the calyx of Held (Dulubova et al., 2005), thus bringing SVs in closer proximity to the release machinery and the VGCCs.

In immature Sept5−/− synapses we observe an unusually low m-value, similar to that observed in mature synapses, indicative of tight VGCC-SV coupling. As above, s-values measured from these synapses correlate with the low m-value and are reduced from those observed in Sept5+/+ synapses of this developmental stage. Finally, via TEM imaging, we found that the SV-AZ separation distribution was shifted leftward in immature Sept5−/− synapses, due to an increased number of docked SVs, confirming that Sept5 is at least involved in the positioning of SVs relative to the AZ and VGCCs. These results provide strong evidence that Sept5 serves to establish increased SV-AZ separation distances in the immature terminal. In addition, immature Sept5−/− synapses appear to have a higher P, and shorter SD than Sept5+/− synapses, independent of AP waveform, which is expected if the SV-VGCC coupling were tighter (Fig. 5.6C). It should be noted however that immature Sept5−/− synapses do not exhibit equivalent quantal release or SD values to those observed in mature synapses even though both m and s values are similar. This suggests that while loss of Sept5 results in tighter coupling between VGCCs and SVs in immature synapses, other factors, such as the precise positioning of VGCCs, perhaps in defined “slots” around the release machinery, may still reside in an immature state (see Cao et al., 2004). This most likely results in a synapse that is intermediate, between normal immature or mature synapses, in terms of its gross functionality.

In contrast, loss of Sept5 in mature synapses appears to have little measurable effect on the localization of SVs or on the electrophysiological markers (m and s) that can be used to infer it.

While the developmental expression levels of Sept5 at the calyx of Held are presently unknown, whole-brain expression has been shown to increase with development (Peng et al., 2002). Why no measurable impact of the loss of Sept5 is observable in mature synapses has a number of potential explanations; Since Sept5 competes with α-SNAP for binding to the 7S complex (Beites et al., 2005), changes in the ratio of expression of either competing protein could alter the distribution of SVs. Levels
of α-SNAP mRNA have been found to increase in rat brain with development, which would decrease the effectiveness of Sept5 binding to syntaxin, decreasing SV-AZ separation on average (Püschel et al., 1994).

While these findings do not confirm variability in the expression levels of either protein at the calyx of Held, they do present a compelling target for future expression assays to address the above questions in this system. Similarly, since Sept5 binds to syntaxin 1A within the 7S complex, a reduction in the amount of syntaxin 1A with development could decrease the likelihood of Sept5 binding and therefore the SV-AZ separation distance. In rat hippocampus, the expression of syntaxin 1 was found to decrease significantly after the first week of postnatal life and stabilize after the second postnatal week (Shimohama et al., 1998). If this expression pattern were true for the calyx of Held, we would expect the binding of Sept5 with syntaxin 1 to be less frequent and SV-AZ separation to tighten. Alternatively, loss of Sept5 may still alter SV localization in mature synapses but to a degree immeasurable by the experimental analyses above. Regardless, it is clear that the developmental expression pattern of many of these proteins must be ascertained at the calyx of Held-MNTB synapse before their impact on SV localization can be resolved.

Alternatively, the expression of Sept5 throughout development may be regulated by translational or post-translational modification. It has been well established that Ca\(^{2+}\) signalling through NMDARs in postsynaptic neurons, in response to repetitive activity, activates a cAMP-PKA-CREB signalling cascade that results in gene translation and protein synthesis necessary for the establishment of long-lasting forms of LTP (Nguyen et al., 1994). PKA-CREB mediated alterations in presynaptic function have also been observed suggesting that similar mechanisms most likely operate to modulate gene transcription in both pre- and postsynaptic elements (Peunova & Enikolopov, 1993). Interestingly, both of these cascades rely on increased Ca\(^{2+}\) as a trigger. Thus, following the onset of activity at the calyx of Held (~P11), increases in Ca\(^{2+}\) influx may activate signalling cascades that alter gene expression subsequently. Sept5 expression may be significantly modified/reduced at this synapse as a result. A translational change in the expression of Sept5 could underlie the lack of observable differences between the functional properties of mature Sept5\(^+/+\) and Sept5\(^{-/-}\) synapses. Moreover, differential phosphorylation levels of CREB during development could further tune Sept5 gene expression. Indeed, lack of sensory activity following cochlear ablation in guinea pig has recently been shown to significantly alter the levels of phosphorylated CREB and alter the types of plasticities observed (Mo et al., 2006).

In addition, post-translational mechanisms may alter the Sept5 protein differentially during development. For example, the presynaptic AZ proteins RIM1 and synapsin are targets for ubiquitination and phosphorylation respectively. Recent studies have shown that the function of both of these proteins is significantly altered depending on their ubiquitination/phosphorylation state (Fiumara et al., 2007; Yao et al., 2007). These or other forms of posttranslational modulation (i.e. sumoylation, etc...) may be developmentally regulated or altered by the increased activity of the calyx of Held-MNTB synapse following the onset of hearing. Currently, it is unknown whether Sept5 is modulated by any of the above means; however, it is clear that a number of potential explanations exist for this protein’s lack of functional impact in the mature synapse.
Our results suggest that Sept5, at least in the immature synapse, plays a substantive role in defining the spatial arrangement of SVs at AZs and thus both $m$ and $s$.

A secondary role for Sept5 containing filaments in tethering SV together has also been proposed. Given that syntaxin-containing 7S complexes exist on SVs (Otto et al., 1997), it is conceivable that Sept5 may be involved in transiting SVs to the AZ as others are depleted. To determine whether Sept5 was involved in this process we evaluated the recovery of both the immediate RRP (IM-RRP) and the intermediate RRP following selective depletion of each subpopulation of SVs. We found no evidence that Sept5 altered the kinetics or extent to which SVs were replenished into either of these pools in either immature or mature synapses (see section 9.2). These results suggest that, while Sept5 may indeed bind between SVs, it has little discernable role in recovery of the RRP. Interestingly, loss of Sept5 did result in increases in the RRP size in both immature and mature synapses (Fig. 5.6). The implications of these findings are described more completely in section 9.2.

The calyx of Held synapse undergoes a series of developmental adaptations which confer its abilities to faithfully reproduce high-frequency signals (Trussell, 1999; Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; von Gersdorff & Borst, 2002; Joshi et al., 2004). One key developmental adaptation which maintains release efficacy, despite a significant reduction in AP width, is the developmental tightening of VGCCs and SVs which, in combination, reduced the $I_{\text{Ca}}$ required for SV release (Fedchyshyn & Wang, 2005; Yang & Wang, 2006). Here we confirm that this adaptation also decreases the duration and dynamic range of SD ensuring minimal temporal jitter and latency in transmission upon AP innervation at the nerve terminal. In addition, we have identified a protein, Sept5, which appears to underlie the increased VGCC-SV separation observed in immature synapses but does not appear to be required for normal replenishment of the RRP in either immature or mature terminals. Interestingly, we did identify a potential novel function for Sept5 in restricting the size of the RRP. While these results are preliminary, they provide an interesting molecular candidate for the functional separation of RRP and RVP.

Taken together, these data show that Sept5 is a key protein in establishing VGCC-SV/AZ separation distances at the immature calyx of Held-MNTB synapse. This adaptation, amongst others, ultimately converges to determine this synapses ability to reproduce high-frequency signals and ensure accurate auditory signal processing.
6. THE IMPACT OF RELEASE MODALITY SWITCHING ON SHORT-TERM PLASTICITY IN THE KINETICS & MAGNITUDE OF SYNAPTIC VESICLE RELEASE
6.1 INTRODUCTION

Plasticity in the output and timing of synapses is crucial for computation and signal processing in the auditory system (Trussell, 1999; Abbott & Regehr, 2004). While the correlations between long-term plasticity (LTP) and learning/memory have received much attention over the several decades since its characterization (Bliss & Lømo, 1973), the true function of short-term plasticity (STP) has been more difficult to elucidate (Abbott & Regehr, 2004). Short-term plasticity (STP) can be defined as activity-dependent transient alterations to the output strength of synapses, which occur on time scales of milliseconds to a few seconds, normally observed at the postsynaptic neuron. These changes in synaptic strength can result presynaptically from changes in the release probability (P_r) of synaptic vesicles (SVs), at a particular release site, arising from a number of possible presynaptic and retrograde postsynaptic mechanisms (reviewed in Zucker & Regehr, 2002; Kushmerick et al., 2004). When P_r is transiently increased or decreased, short-term facilitation (STF) or short-term depression (STD) is observed in synaptic output respectively (Zucker & Regehr, 2002).

At the calyx of Held-MNTB synapse, STP has been particularly well studied owing to the synapses unique morphology allowing for electrophysiological access to both pre- and postsynaptic elements of the synapse (Forsythe, 1994; Borst et al., 1995; Takahashi et al., 1996). Moreover, the rapid and well characterized development of the synapse has allowed for STP to be studied in the alternative context of conferring this synapses ability to accurately follow high-frequency input (reviewed in von Gersdorff & Borst, 2002; Schneggenburger et al., 2002; Xu et al., 2007).

STD can be attributed to factors residing in both pre- and postsynaptic elements of the synapse, the composition of which depends on the activity level (input frequency) and the particular system being observed (Zucker, 1989; Zucker & Regehr, 2002; von Gersdorff & Borst, 2002). Presynaptically, activity-dependent depletion of SVs (reviewed in Schneggenburger et al., 2002; Weiss et al., 1999; Wu & Borst, 1999; Sakaba & Neher, 2001; Sun & Wu, 2001), Ca^{2+}-dependent inactivation of the Ca^{2+} sensor (Hsu et al., 1996; Zucker, 1996; Dobrunz et al., 1997), inactivation of VGCCs (Xu & Wu, 2005), activation of presynaptic metabotropic glutamate receptors (mGluRs) (von Gersdorff et al., 1997; Billups et al., 2005), and decreases in the sensitivity of the release machinery to Ca^{2+} (Wölfel et al., 2007) all contribute to depression of observed postsynaptic responses (I_{EPSC}). Postsynaptically, AMPAR desensitization, most prominent in immature synapses (Trussell et al., 1993; Otis et al., 1996; Wong et al., 2003; Scheuss et al., 2002) and AMPAR saturation (Sun & Wu, 2001; Chen et al., 2002; Scheuss et al., 2002) are amongst the most common sources of STD.

STF is thought to originate exclusively from the presynaptic terminal, but its effect on synaptic output is often masked by STD occurring concurrently through any of the mechanisms described above (Dittman et al., 2000; von Gersdorff & Borst, 2002). The most prominent theory describing STF is the residual Ca^{2+} hypothesis in which elevations in [Ca^{2+}], from prior VGCC openings, prime the SV release machinery transiently increasing P_r at those sites for subsequent release events (Katz & Miledi, 1968; Katz, 1969; reviewed in Zucker & Regehr, 2002; Kamiya & Zucker, 1994; Dittman et al., 2000). However, the mechanism by which Ca^{2+} ions act to increase P_r has been the subject of much debate.
Initial hypotheses postulated that the Ca\(^{2+}\) ions, responsible for STF, bind to the same Ca\(^{2+}\) sensor that underlies phasic SV release (Katz & Miledi, 1968; Katz, 1969). Subsequent investigation suggested that a second Ca\(^{2+}\) sensor, not directly involved in release of SVs, may function cooperatively to facilitate release (Atluri & Regehr, 1996; reviewed in Burnashev & Rozov, 2005). At the calyx of Held-MNTB synapse, neuronal Ca\(^{2+}\) sensor 1 (NCS-1) was put forth as a candidate for this “facilitation sensor” (Tsujimoto et al., 2002). Saturation of endogenous Ca\(^{2+}\) buffers has also been suggested as underlying the rise in [Ca\(^{2+}\)]\(_{\text{Bi}}\), observed during prolonged synaptic activity (Felmy et al., 2003). Finally, \(I_{\text{Ca}}\) themselves have been shown to facilitate in response to certain stimulation frequencies, however, it is arguable how much of the synaptic facilitation (\(I_{\text{EPSC}}\)) comes as a result (Xu & Wu, 2005; Inchaupse et al., 2004; Ishikawa et al., 2005; Felmy et al., 2003). Despite the many lines of evidence explaining the process of STF, the actual extent to which each, or any of these mechanisms, contribute to the STP observed in synapses during repetitive activity is unknown (Zucker & Regehr, 2002; Burnashev & Rozov, 2005).

Further complicating understanding of STP at the calyx of Held-MNTB synapses is the observation that the patterns of STP change with maturation, showing reduced STD and a concurrent decrease in \(P_r\), resulting from a number of developmental adaptations (Joshi & Wang, 2002; Taschenberger et al., 2002; Fedchyshyn & Wang, 2005; Yang & Wang, 2006).

From the findings above, two principles appear common in reconciling the mechanisms underlying STP at the calyx of Held-MNTB synapse. First, the principle of heterogeneity in \(P_r\) is one requirement that emerges from most models describing STF/STD (Meinrenken et al., 2002; Meinrenken et al., 2003; Trommershäuser et al., 2003; Wadel et al., 2007; Wölfel et al., 2007; Wu & Borst, 1999; Sakaba & Neher, 2001; Sakaba et al., 2002). However, the specific source of the heterogeneity remains largely debated. Second, factors affecting Ca\(^{2+}\), either within ([Ca\(^{2+}\)]) or entering the nerve terminal (\(I_{\text{Ca}}\)), will be amplified, by the non-linearity of Ca\(^{2+}\) binding to the Ca\(^{2+}\) sensor (Dodge & Rahamimoff, 1967) and the non-linear Ca\(^{2+}\)-channel/domain cooperativity required to provide a supra-threshold Ca\(^{2+}\) signal for release (Fedchyshyn & Wang, 2005), to have significant effects on synaptic output and \(P_r\). These changes in \(P_r\), not only have profound effects on synaptic strength but may also impact the kinetics of release and the fidelity of synaptic transmission (see Chapter 5; Wadel et al., 2007; Hermann et al., 2007; Fedchyshyn & Wang, 2007; Lin & Faber, 2002; Vyshedskiy et al., 2000; Waldeck et al., 2000).

Using simultaneous pre- and postsynaptic whole-cell electrophysiological recordings, from immature (P8-12) and mature (P16-18) calyx of Held-MNTB synapses, we investigated the effects of release modality tightening on the patterns of STP observed at the calyx of Held (Fedchyshyn & Wang, 2005). By using trains of real APs (200Hz for 200ms) of varying widths, as presynaptic voltage-commands, we controlled for changes in \(P_r\), associated with the developmental narrowing of presynaptic APs and the associated reduction in \(I_{\text{Ca}}\) observed at this synapse. Employing this technique, using the slow Ca\(^{2+}\) buffer EGTA, and immature Sept5\(^{-/-}\) synapses, which have an unusually tight effective VGCC-SV coupling, we were able to assay how differences in VGCC-SV coupling impact \(P_r\) and the patterns of STP in both synaptic timing and strength.

Here, we propose and provide evidence for a model that considers loose VGCC-SV coupling as underlying heterogeneity in \(P_r\) and for creating conditions permissible to STF at the immature calyx of
Held-MNTB synapse. We also suggest that a decrease in SD during STF, and the STF itself, are the result of both $\text{Ca}^{2+}$-dependent priming of the release machinery and facilitation of $I_{\text{Ca}}$ at this developmental stage.

In contrast, the tightly-coupled arrangement of VGCCs and SVs found in mature synapses, results in a more homogeneous population of VGCC-SV units in which STF, through $\text{Ca}^{2+}$-dependent priming, is much less likely to occur. In both immature and mature synapses, STD was found to be the result of decreases in $P$, caused by SV depletion and the recruitment of SVs distant from the AZs, which was accompanied by an increase in SD.

Taken together, these results suggest that VGCC-SV coupling determines, at least in part, the patterns of STP in both timing and synaptic strength observed at both the immature and mature calyx of Held-MNTB synapse. Moreover, the decrease in the range of STP patterns observed, both in terms of timing and synaptic strength, in mature synapses occurs in response to the transformation from a microdomain to nanodomain release modalities during development. This adaptation may provide a means by which the calyx of Held-MNTB synapse consolidates its ability to transmit high-frequency signals with high-reproducibility and reliability.
6.2 RESULTS

6.2.1 Identical Input Waveforms Evoke Distinctive Patterns of Postsynaptic Responses in Immature & Mature Synapses.

At the calyx of Held, many concurrent changes occur both pre- and postsynaptically during development (Taschenberger & von Gersdorff, 2000, Taschenberger et al., 2002, Joshi & Wang, 2002, Joshi et al., 2004, Fedchyshyn & Wang, 2005, Yang & Wang, 2006). Many of these changes have been well characterized; however, characterization of steps late in the neurotransmission cascade can be complicated by developmental changes occurring upstream. To investigate how the spatial coupling of voltage-gated Ca\textsuperscript{2+} channels (VGCCs) and synaptic vesicles (SVs) impacts synaptic output during high-frequency activity across development, changes occurring in the width and kinetics of the action potential (AP) waveform during development negate the use of simple afferent-evoked stimulation. To control for developmental changes in the synaptic input waveform, we recorded trains of real APs from current-clamped calyces in response to afferent evoked stimulation (200Hz, 200ms) (Fig. 6.1A). AP trains were recorded from an immature calyx (AP\textsubscript{I}, P10), a mature calyx (AP\textsubscript{M}, P16), and a mature calyx at 35\textdegree C (AP\textsubscript{35}, P16) which provided individual APs with half-widths of approximately 0.55ms, 0.39ms, and 0.24ms respectively (measured at –30mV), inline with those expected for each recording condition and developmental stage (Fig. 6.1A, right panel) (Taschenberger & von Gersdorff, 2000). All AP trains were scaled to have identical amplitudes (130mV, measured from -80mV) to ensure that the driving force for Ca\textsuperscript{2+} was controlled across the different waveforms, leaving only the variable AP widths to determine the magnitude of calcium current (I\textsubscript{Ca}) influx in the nerve terminal. The three AP trains were then used as voltage-clamp command waveforms to evoke I\textsubscript{Ca}, and the corresponding excitatory postsynaptic currents (I\textsubscript{EPSC}), from immature (P8-12) and mature (P16-18) synapses in 1mM [Ca\textsuperscript{2+}]\textsubscript{o} (King & Meriney, 2005; see section 2.2.5).

In both immature and mature synapses, the widest AP\textsubscript{I} waveform generated the largest I\textsubscript{Ca}, with narrowed AP waveforms (i.e. AP\textsubscript{M} & AP\textsubscript{35}) generating progressively smaller I\textsubscript{Ca} (Fig. 6.1B,C). I\textsubscript{Ca} amplitudes were similar between immature and mature groups, for any given AP waveform, suggesting that VGCCs in either developmental stage were equally responsive to the voltage command APs and were generating similar input signals for a given AP. Despite similar I\textsubscript{Ca}, the I\textsubscript{EPSC} patterns were vastly different across developmental groups with immature synapses exhibiting stronger facilitation and smaller I\textsubscript{EPSC} for all AP waveforms (Facilitation Index: 50±6 vs. 2±1%, AP\textsubscript{M}; 25±8 vs. 0±0%, AP\textsubscript{I}; immature vs. mature) (Fig. 6.4A). In both test groups, I\textsubscript{EPSC} facilitation occurred only during the early events of the trains, however, only the narrowest AP\textsubscript{35} trains triggered significant facilitation in mature synapses, while both the AP\textsubscript{I} and AP\textsubscript{M} APs triggered progressively greater I\textsubscript{EPSC} facilitation in immature synapses (Fig. 6.1B,C). Following the initial events in each stimulus train, synaptic depression dominated the patterns of short-term plasticity (STP) in I\textsubscript{EPSC} for all AP waveforms and both developmental groups (Fig. 6.1B,C). When the AP\textsubscript{35} waveform was used to drive I\textsubscript{Ca} in immature synapses, reliable synaptic output could not be attained, with many AP events failing to evoke any
Figure 6.1: Developmental Dependence of Synaptic Output on Action Potential Waveform.

(A) Trains of afferent-evoked APs (200Hz, 40 events), recorded from an immature (AP_{i}), mature (AP_{m}), and mature calyx at 35°C (AP_{35}), superimposed, and scaled to the same amplitude (120mV). Boxed region is magnified at right. (B) Presynaptic I_{Ca} and postsynaptic I_{EPSC} recorded from an immature calyx in response to presynaptic voltage commands shown in A (1mM [Ca^{2+}]). Records in response to AP_{i} (top panel), AP_{m} (middle panel), and AP_{35} (bottom panel) are recorded from the same P11 synapse. (C) As in B recorded from a single P18 synapse. (D) Pooled data of I_{EPSC} amplitude, normalized to the maximal I_{EPSC}, against event number for immature synapses in response to AP_{i} (closed circles, n=8) and AP_{m} (closed squares, n=9) input waveforms. Note that reliable transmission could not be achieved from immature synapses in response to the AP_{35} waveform. (E) As in D for mature synapses.
measurable SV release (Fig. 6.1B, bottom panel). However, the same input waveform evoked reliable transmission from mature terminals, in line with our previous findings (Fedchyshyn & Wang, 2005). This suggests that the tighter spatial coupling of VGCCs and SVs in mature synapses may account for the developmental difference in synaptic efficacy in response to the same input waveforms (Fig. 6.1C, bottom panel). In addition, mature synapses showed more rapid depression of I_{EPSC}, than did immature synapses in response to a given AP train, however, the overall extent of synaptic depression was not significantly different between immature and mature synapses for either the AP_{M} or AP_{I} waveforms (Fig. 6.1D,E, 6.4B).

The rapid depression and increased synaptic efficacy observed in mature synapses implies that these synapses have a higher release probability (P_{r}) at each active zone (AZ) than do immature synapses for a given input (I_{Ca}). This is likely due, at least in part, to the reduced requirement for many VGCCs to act in a cooperative manner in releasing SVs. In contrast, the loose coupling of VGCCs and SVs in immature synapses decreases the likelihood that an AP will provide a sufficient Ca^{2+} signal, in the neighbourhood of a SV, to trigger its release.

Thus we hypothesize that, as a by-product of their reduced P_{r} for a given AP waveform, loosely-coupled VGCC-SV units in immature synapses are more likely to experience sub-threshold Ca^{2+} signals upon AP innervation. This Ca^{2+} influx contributes to elevating residual [Ca^{2+}] which may prime SVs, facilitating their release in response to subsequent APs (Fig. 6.1B,D). Given the above, we sought to link changes in P_{r}, brought about by distinct modalities of VGCC-SV coupling, to the patterns of short-term plasticity (STP) in I_{EPSC} observed.

6.2.2 Increasing the Driving Force For Ca^{2+} Shifts the Patterns of Short-Term Plasticity to Favour Depression in Both Immature & Mature Synapses.

Using cooperativity (m) as a measure of the effective spatial coupling of VGCCs and SVs, we asked whether reducing m, by increasing [Ca^{2+}]_{o} from 1 to 2mM, would result in patterns of STP indicative of the resulting elevated P_{r}. Increasing [Ca^{2+}]_{o} increases the size, penetration, and spread of [Ca^{2+}] domains and drives m lower, increasing the probability that a given AP will provide a sufficient Ca^{2+} signal for engagement of the release machinery (Gentile & Stanley, 2005, Fedchyshyn & Wang, 2005). Since SVs that have been released are unavailable for Ca^{2+}-dependent priming, the propensity for facilitation is reduced, resulting in more rapid depletion of the RRP and STD (Oleskevich et al., 2000, Brenowitz & Trussell, 2001). Thus we expect that increasing [Ca^{2+}]_{o} to 2mM should produce STP patterns that are reflective of the increased P_{r}, brought about by a reduction in m, for a given AP waveform.

Using the AP waveforms shown in Fig. 6.1A, we evoked I_{Ca} from immature synapses in 2mM [Ca^{2+}]_{o} (Fig. 6.2A). In response to the wide AP_{I} waveforms, immature synapses showed strong synaptic depression with no facilitation observed during the AP trains (Fig. 6.2A,C). This is in contrast to the strong facilitation observed in response to the same APs in 1mM [Ca^{2+}]_{o} (Fig. 6.1B, top panel). In immature synapses AP_{M} waveforms evoked I_{EPSC} trains with some early facilitation, similar in form to the
Figure 6.2: Increasing [Ca\(^{2+}\)]\(_o\) Decreases Synaptic Facilitation Independent of AP Waveform.

(A) Presynaptic I\(_{Ca}\) and postsynaptic I\(_{EPSC}\) recorded from an immature calyx in response to presynaptic voltage commands using the AP trains shown in Fig. 6.1A (2mM [Ca\(^{2+}\)]\(_o\)). Records in response to AP\(_o\) (top panel), AP\(_M\) (middle panel), and AP\(_{35}\) (bottom panel) are recorded from the same P11 synapse. (B) Presynaptic I\(_{Ca}\) and postsynaptic I\(_{EPSC}\) recorded from a mature calyx in response to presynaptic voltage commands using the AP trains shown in Fig. 6.1A (2mM [Ca\(^{2+}\)]\(_o\)). Records in response to AP\(_o\) (top panel), AP\(_M\) (middle panel), and AP\(_{35}\) (bottom panel) are recorded from the same P18 synapse. (C) Pooled data of I\(_{EPSC}\) amplitude, normalized to the maximal I\(_{EPSC}\), against event number for immature synapses in response to AP\(_o\) (closed circles, n=5), AP\(_M\) (closed squares, n=5), and AP\(_{35}\) (closed triangles, n=5) input waveforms. (D) Pooled data of I\(_{EPSC}\) amplitude, normalized to the maximal I\(_{EPSC}\), against event number for mature synapses in response to AP\(_o\) (open circles, n=5), AP\(_M\) (open squares, n=5), and AP\(_{35}\) (open triangles, n=5) waveforms.
I_{EPSC} triggered by the wider APs trains in 1mM [Ca^{2+}]_o (Fig. 6.2A middle panel vs. Fig 6.1B top panel). With the increase in [Ca^{2+}]_o, immature synapses now responded faithfully to all AP_{35} trains, generating I_{EPSC} patterns similar to those observed in mature synapses in 1mM [Ca^{2+}]_o in response to the same APs (Fig. 6.1C, 6.2B, bottom panels).

Mature synapses exhibited strong depression in I_{EPSC} in response to all but the narrowest AP_{35} waveforms, which triggered moderate I_{EPSC} facilitation during the early events of the train (Fig. 6.2B). Facilitation indices were 65±1% vs. 29±9% for AP_{35}; 32±8% vs. 0±0% for AP_{35}; and 1±1% vs. 0±0% for AP_{1} trains in immature and mature synapses respectively (Fig. 6.4A). Depression indices were 40±8% vs. 76±3% for AP_{35}; 93±1% vs. 89±2% for AP_{35}; and 98±0.5% vs. 92±1% for AP_{1} trains in immature and mature synapses respectively (Fig. 6.4B). Despite the increase in [Ca^{2+}]_o, immature synapses still showed stronger facilitation in I_{EPSC} than did mature synapses for any of the input waveforms (facilitation index, Student’s t-test, p<0.05) suggesting that the P_i in these synapses is still lower than that of mature synapses at 2mM [Ca^{2+}]_o for a given AP waveform.

Of note is that the differences in the patterns of STP, between immature and mature synapses, were less pronounced in 2mM [Ca^{2+}]_o than those observed in 1mM [Ca^{2+}]_o. This is consistent with the smaller difference in m-values, observed between immature and mature synapses, upon increasing [Ca^{2+}]_o, if the difference in P_i observed above is the result of differences in the effective coupling of VGCCs and SVs between experimental groups (Fig. 6.1B,C, 6.2A,B) (Fedchyshyn & Wang, 2005). Interestingly, the patterns of STP observed in immature synapses, in 2mM [Ca^{2+}]_o, were similar to those observed in mature synapses at 1mM [Ca^{2+}]_o. This is again consistent given the similar m-values under these two conditions (Fig. 6.2C, 6.1E).

These results suggest that the degree Ca^{2+}-domain cooperation/overlap required to provide a supra-threshold Ca^{2+} signal, is a highly influential component in the culmination of stochastic processes that determine P_i and STP. So while the duration and amplitude of an AP defines the open probability (P_o) and driving force for Ca^{2+} through VGCCs, the VGCC-SV coupling determines whether the sum of open VGCCs, in the vicinity of an SV, will provide a sufficient Ca^{2+} signal to trigger its release. Thus, factors which affect m, such as VGCC-SV spatial separation or [Ca^{2+}]_o, will have profound effects on the P_i of a SV and the patterns of STP observed.

6.2.3 Immature, Low-m Synapses Exhibit Slight Alterations in STP Patterns Toward Those Observed in Other Low-m Systems.

In further exploring the link between VGCC-SV coupling and the patterns of STP in I_{EPSC}, we made use of two experimental conditions, in immature synapses, which produce low effective m-values. By loading immature (high-m) presynaptic terminals with 10mM EGTA, loosely-coupled VGCC-SV units are functionally excluded from participating in SV release events due to the interception of Ca^{2+} ions in transit between the VGCC and the release machinery by the buffer (Adler et al., 1991; Borst & Sakmann, 1996; Fedchyshyn & Wang, 2005). Those VGCC-SV units that underlie the remaining quantal output therefore must be of the tightly-coupled (low-m) variety, resulting globally in a low-m synapse.
Figure 6.3: Reductions in Cooperativity Have Negligible Effects on the Patterns of Short-Term Plasticity.

(A) Presynaptic $I_{\text{Ca}}$ and postsynaptic $I_{\text{EPSC}}$ from an immature synapse, loaded presynaptically with 10mM EGTA, in response to AP, (top panel) and AP$_u$ (bottom panel) waveforms (1mM [Ca$^{2+}$]). (B) As in A recorded from an immature Sept5$^+$ synapse. (C) Pooled data of $I_{\text{EPSC}}$ amplitude, normalized to the maximal $I_{\text{EPSC}}$, against event number for immature synapses (closed squares, from Fig. 6.1D) and immature synapses loaded presynaptically with 10mM EGTA (dark grey filled squares) in response to the AP$_u$ waveform. (D) As in C for immature Sept5$^+$ (light grey filled squares) and WT (filled squares) synapses in response to the AP$_u$ waveform. (E) Pooled data of $I_{\text{EPSC}}$ amplitude, normalized to the maximal $I_{\text{EPSC}}$, against event number for immature synapses (closed squares, from Fig. 6.1D) and immature synapses loaded presynaptically with 10mM EGTA (dark grey filled squares) in response to the AP$_\text{WT}$ waveform. (F) As in E for immature Sept5$^+$ (light grey filled squares) and WT (filled squares) synapses in response to the AP$_\text{WT}$ waveform.
We also took advantage of the unusually low $m$-value measured in immature Sept5−/− synapses, which we have hypothesized to be the result of altered VGCC-SV coupling (see Chapter 5). If the observed patterns of STP are linked to $P_o$ through $m$, as described above, we hypothesize that both of these low-$m$ synapses should exhibit STP patterns reminiscent of other low-$m$ conditions (i.e. mature synapses, 2mM $[\text{Ca}^{2+}]_o$). Thus we expect that immature synapses, either following loading of 10mM presynaptic EGTA or in the absence of the Sept5 protein, should have less facilitation than is observed in untreated, wild-type (WT) immature synapses.

To investigate the patterns of STP in artificial low-$m$ conditions, we loaded immature calyces with 10mM EGTA and inputted either the AP$_m$ or AP$_i$ waveforms as presynaptic voltage-commands in 1mM $[\text{Ca}^{2+}]_o$ (Fig. 6.3A). Although presynaptic loading of EGTA generally attenuated $I_{\text{EPSC}}$, in agreement with our previous findings, the patterns of STP were only marginally affected by addition of the Ca$^{2+}$ buffer (Fig. 6.3C,E). In comparison to immature synapses without the addition of EGTA, EGTA loaded synapses showed a significantly lower facilitation index, in response to either the AP$_m$ or AP$_i$ waveforms (26±5% vs. 50±6% for AP$_m$, 6±3% vs. 25±8% for AP$_i$) (Fig. 6.3C,E, 6.4A). This suggests that, while EGTA-loaded synapses release fewer quanta per AP, those quanta have a higher $P_o$ than do quanta from unloaded synapses of the same developmental stage. Thus addition of EGTA seems to reduce the facilitation observed in high-$m$ synapses to a level similar to that which is observed when $m$ is reduced by raising $[\text{Ca}^{2+}]_o$ to 2mM (Fig. 6.4A).

When we measured the depression index, we found that presynaptic loading of EGTA had no effect on the degree of depression observed in immature synapses in response to the AP$_m$ waveform, with depression indices of 83±2% vs. 79±2% for EGTA unloaded and loaded synapses respectively (Fig. 6.4B). In response to the wider AP$_i$ waveforms, a small but significant difference in depression index was observed between EGTA loaded and unloaded synapses, with depression indices of 93±1% vs. 88±1% for EGTA unloaded and loaded synapses respectively. These data suggest that the addition of EGTA increases the relative steady-state amplitude reached later in the AP trains by altering the balance between replenishment rate of the RRP and the depletion of SVs during the AP$_i$ trains (Fig. 6.3E, 6.4B). This difference is most-likely due to a reduction in the depletion rate of the RRP, caused by a reduction in the build-up of high concentrations of intra-terminal Ca$^{2+}$ during the trains. While presynaptic addition of 10mM EGTA did attenuate facilitation in immature synapses for both AP$_m$ and AP$_i$ waveforms, it did not completely abolish facilitation, as observed in mature synapses in response to the same AP waveforms. This suggests that, while alterations in $m$ have a clear impact on the patterns of STP, other aspects of synaptic transmission, that change during development, also contribute to forming the patterns of STP observed in the mature synapse.

Using immature Sept5−/− synapses, we similarly used the AP$_m$ and AP$_i$ waveforms to generate $I_{\text{Ca}}$ and $I_{\text{EPSC}}$ from this low-$m$ system (Fig. 6.3B). When we compared the patterns of STP, little difference was observed between Sept5−/− and WT synapses in response to either the AP$_m$ or AP$_i$ trains (Fig. 6.3D,F). Accordingly, facilitation and depression indices showed no significant difference between the two test groups. Facilitation indices were 50±6% vs. 43±9% for AP$_m$ and 25±8% vs. 18±4% for AP$_i$. 

In untreated, wild-type immature synapses, 2mM $[\text{Ca}^{2+}]_o$ attenuated facilitation from immature synapses of the same developmental stage. Thus addition of EGTA seems to reduce the facilitation observed in high-$m$ synapses to a level similar to that which is observed when $m$ is reduced by raising $[\text{Ca}^{2+}]_o$ to 2mM (Fig. 6.4A).
Figure 6.4: Facilitation & Depression Indices in High & Low Cooperativity (m) Synapses.  
(A) Pooled data showing facilitation index of $I_{EPSC}$ in response to each of AP$_{35}$, AP$_{M}$, and AP$_{I}$ waveforms, for all experimental conditions in which reliable synaptic transmission was recorded.  
(B) As in A, showing pooled data for depression index.
trains in WT and Sept5\textsuperscript{-/-} synapses respectively (Fig. 6.4A). Depression indices were 83\% \pm 2\% vs. 76\% \pm 5\% for AP\textsubscript{M} and 93\% \pm 1\% vs. 90\% \pm 1\% for AP\textsubscript{I} trains in WT and Sept5\textsuperscript{-/-} synapses respectively (Fig. 6.4B).

The absence of a significant effect on STP, given a loss of Sept5, implies that, despite its apparent role in defining VGCC-SV placement at the active zone (AZ), Sept5 has a relatively insignificant role in shaping the patterns of STP in immature synapses. In immature Sept5\textsuperscript{-/-} synapses it is likely that, while there may be more SVs docked at the AZ, the arrangement of VGCCs around those SVs has not yet matured resulting in an incomplete “conversion” of release modality and the STP patterns expected of their low-m. Alternatively, Sept5 may fulfil secondary roles in the nerve terminal that may act in opposition to the tighter coupling observed in these synapses during repetitive stimulation. Indeed, we have found that the loss of Sept5 significantly increases the size of the available pool of SVs (see section 9.2) which could act to provide extra SVs to the terminal and alter the patterns of STP in a manner contrary to that expected from the reduced m. Despite this, and other possibilities, the lack of an observable difference in the patterns of STP between WT and Sept5\textsuperscript{-/-} synapses suggests that the patterns of STP, observed at a particular developmental stage, may depend on factors in addition to the spatial coupling of VGCCs and SVs.

While our above experiments; using different AP waveforms in 1 and 2mM [Ca\textsuperscript{2+}], and through presynaptic loading of EGTA, support the hypothesis that m, at least in part, determines the patterns of STP through affects on P\textsubscript{r}, our data from Sept5\textsuperscript{-/-} synapses suggest further investigation regarding the effects of this deletion is necessary. While it is clear that there are many factors that may influence the patterns of STP, such as P\textsubscript{r}, the availability of SVs, and the sensitivity of the release machinery to Ca\textsuperscript{2+}, it is not yet clear how the loss of Sept5 influences these factors and other aspects of the SV cycle (Beites \textit{et al.}, 1999; Beites \textit{et al.}, 2005). Having previously linked VGCC-SV coupling to changes in the kinetics of SV release during single AP events (see Chapter 5) we next sought to determine whether the patterns of synaptic delay (SD) during repetitive activity were also affected by differences in m.

6.2.4 Bidirectionality in Synaptic Delay Occurs Only During Periods of Strong Synaptic Facilitation.

Having characterized the phenomenon of activity-dependent prolongation in SD in the juvenile calyx of Held synapses (Fedchyshyn & Wang, 2007), we sought to determine whether the differences in P\textsubscript{r}, attributed to changes in VGCC-SV coupling would also affect plasticity in SD. Using the I\textsubscript{Ca}/I\textsubscript{EPSC} generated from immature and mature synapses, in response to the various AP waveforms, we measured the SD for each event of the train in 1mM [Ca\textsuperscript{2+}]. We then split the first 20 events of the trains into an early (first 5 events) and late (next 15 events) phase, to which we applied a least squares linear fit to determine the rate of change, or progression, of SD per event (s\textsubscript{E} \& s\textsubscript{L}).

When we compared the progression of SD, in response to the AP\textsubscript{M} waveform, we found that mature synapses exhibited a monotonic increase in their synaptic delay in agreement with previous findings near this developmental stage (s\textsubscript{E}=6.1\% \pm 0.4, s\textsubscript{L}=1.3\% \pm 0.3) (Fig. 6.5A,C) (Fedchyshyn & Wang, 2007). However, when we examined the SD progression of immature synapses in response to the same AP\textsubscript{M} input, we found that SD decreased in approximate correlation with the period of facilitation (~first 5
Figure 6.5: Bi-Directionality in Synaptic Delay Occurs in Parallel With Synaptic Facilitation & Depression in Immature Synapses.

(A) Presynaptic $I_{\text{Ca}}$ and postsynaptic $I_{\text{EPSC}}$ from the immature (top panel) and mature (bottom panel) synapse shown in Fig. 6.1B, in response to the AP$_{\text{M}}$ waveform (1mM [Ca$^{2+}$]). Three boxed regions, around the first (black), fifth (red), and final (blue) events are magnified and aligned by $I_{\text{Ca}}$ peak at right. Heavy black dashed line denotes time of $I_{\text{Ca}}$ peaks and thin dashed lines denote the timing of $I_{\text{EPSC}}$ onset.

(B) Pooled data of SD, normalized to the SD of the first event, against event number for the complete train (top panel) and the first 20 events (bottom panel), recorded from immature synapses in response to AP$_{i}$ and AP$_{M}$ waveforms (1mM [Ca$^{2+}$]). Least-squares linear regression lines are fitted to the first 5 data points and to points 6 to 20 (bottom panel) with slope of each segment shown in legend.

(C) As in B for mature synapses in response to AP$_{i}$, AP$_{M}$, and AP$_{35}$ waveforms.
events), then increased monotonically during the remaining events of the train ($s_E=-2.1\pm0.7$, $s_L=0.7\pm0.1$) (Fig. 6.5A,B). In contrast, both immature and mature synapses exhibited unidirectional SD progression in response to the broader AP$_i$ waveform, as would be expected if the progression of SD were correlated to the observed pattern of STP in $I_{EPSC}$ ($s_E=0.8\pm0.5$, $s_L=1.3\pm0.3$ vs. $s_E=13.9\pm1.3$, $s_L=1.0\pm0.4$) (Fig. 6.5B,C). In response to the narrowest AP$_{35}$ waveforms, to which only mature synapses responded reliably, mature synapses exhibited a slight period during which SD was constant followed by a modest increase during the remaining events ($s_E=-0.8\pm1.2$ not different from 0, $s_L=0.5\pm0.1$, Student’s t-test, $p<0.05$) (Fig. 6.5C). Interestingly, following the initial change in SD over the first 5 events, only immature synapses showed significantly different late progression rates of SD for the different APs (0.7±0.1 for AP$_M$ and 1.3±0.3 for AP$_i$, Student’s t-test, $p<0.05$), while mature synapses showed similar rates of SD increase during this same period (0.5±0.1, 1.3±0.3, and 1.0±0.5 for AP$_{35}$, AP$_M$, and AP$_i$ respectively, $p>0.05$) (Fig. 6.5B,C, 6.9A,B). Besides the progression rate of SD, the magnitude of increase in SD, observed throughout the AP trains, also depended on the width of the AP waveform in both immature and mature synapses. The wide AP$_i$ trains generated the largest relative increase in SD while the AP$_M$ or AP$_{35}$ trains generated more modest increases, with the overall increase in SD being determined largely by changes in SD occurring during the initial (~5) events (Fig. 6.5B,C, 6.9A,B).

These data suggest that differences in AP waveform, and thus $I_{Ca}$, impact the patterns of both STP in $I_{EPSC}$ and in SD most strongly during the initial few events in a train, after which differences in the observed patterns generally decrease. Interestingly, mature synapses, in response to the wide AP$_i$ trains, showed some recovery of their increased SD late (~event 30) in the AP trains (Fig. 6.5C). The fact that this recovery is only observable in response to the widest AP waveforms, and that mature synapses show an increased readily releasable pool (RRP) size when APs are broadened with TEA (see section 5.2.11), suggests that recovery of SD, late in trains, could be determined by an increased availability of SVs and/or the rate at which they are replenished as is the case for recovery from depression in $I_{EPSC}$ (Wang & Kaczmarek, 1998). Moreover, as was the case for $I_{EPSC}$ facilitation, immature synapses appear more prone to reductions in SD given a particular AP waveform. The directionality of SD progression, which we observed early in trains, seemed to correlate approximately with the occurrence of facilitation or depression during the same period.

Given the above proposed link between the patterns of STP in $I_{EPSC}$, $P_n$, and VGCC-SV coupling, we hypothesized that bidirectionality in SD may also arise from this link. By this logic, the same transient increases in $P_n$ that promote $I_{EPSC}$ facilitation in high-$m$ synapses, may also speed the release kinetics of primed SVs thereby shortening SD. In contrast, prolongations in SD, occurring later in trains, could arise from depletion of the RRP and the additional time required to transit SVs to the AZ from more distant locations. Alternatively, increases in intra-terminal $[Ca^{2+}]_o$ could result in desensitization of the $Ca^{2+}$ sensor or release machinery which may result in a net decrease in the rate of SV release. In either case, if the direction of SD progression depends on $P_n$, and $P_r$ depends on the coupling of VGCCS and SVs, then increasing $[Ca^{2+}]_o$ should also increase $P_r$, lower $m$ in both immature and mature synapses, and decrease the likelihood of both $I_{EPSC}$ facilitation (as observed in section 6.2.2) and shortening of SD.
Figure 6.6: Decreasing the Requirement for Ca\(^{2+}\)-Domain Overlap Eliminates the Bi-Directionality in Synaptic Delay in Parallel With a Decrease in Synaptic Facilitation.

(A) Presynaptic I\(_{\text{Ca}}\) and postsynaptic I\(_{\text{EPSC}}\) from the immature (top panel) and mature (bottom panel) synapse shown in Fig. 6.2A, in response to AP\(_{\text{i}}\) waveform (2mM [Ca\(^{2+}\)]). Three boxed regions, around the first (black), fifth (red), and final (blue) events are magnified and aligned by I\(_{\text{Ca}}\) peak at right. Heavy black dashed line denotes time of I\(_{\text{Ca}}\) peak and thin dashed lines denote the timing of EPSC onset. (B) Pooled data of SD, normalized to the SD of the first event, against event number for the complete train (top panel) and the first 20 events (bottom panel), recorded from immature synapses in response to AP\(_{\text{i}}\), AP\(_{\text{M}}\), and AP\(_{\text{35}}\) waveforms (2mM [Ca\(^{2+}\)]). Least-squares linear regression lines are fitted to the first 5 data points and to points 6 to 20 (bottom panel) with slope of each segment shown in legend. (C) As in B recorded from mature synapses.
To address this hypothesis, we investigated the patterns of SD progression in 2mM [Ca\(^{2+}\)\(_o\)] in response to AP\(_{35}\), AP\(_{M}\), and AP\(_{I}\) waveforms. Upon increasing [Ca\(^{2+}\)\(_o\)], the bidirectionality in SD progression, observed previously in immature synapses, in response to the AP\(_{M}\) waveform was lost (Fig. 6.6A, top panel). In response to these APs, mature synapses also showed unidirectional progression of SD during the trains which was significantly stronger early in trains than in immature synapses (Immature: \(s_E=1.1\pm 1.0\), \(s_L=-1.8\pm 0.5\) vs. Mature: \(s_E=11.4\pm 1.1\), \(s_L=0.6\pm 0.4\), p<0.05) (Fig. 6.6A, 6.9A,B). In immature synapses, bidirectionality in SD progression was now observable in response to the narrowest AP\(_{35}\) trains, but was absent from mature synapses under the same conditions (Immature: \(s_E=-5.8\pm 2.3\), \(s_L=-0.4\pm 0.3\) vs. Mature: \(s_E=1.2\pm 0.9\), \(s_L=0.7\pm 0.2\)) (Fig. 6.6B,C). Finally, in response to the widest AP\(_{I}\) waveforms, both immature and mature synapses showed strong increases in SD progression early in AP trains, in addition to those observed in 1mM [Ca\(^{2+}\)\(_o\)] during the same period (Immature: \(s_E= 3.7\pm 0.2\) and Mature: \(s_E=23\pm 3\)) (Fig. 6.6B,C, 6.9A,B). As was the case in 1mM [Ca\(^{2+}\)\(_o\)], immature synapses showed significantly greater variability in the late phase of SD progression between different AP trains than did mature synapses, with mature synapses again showing recovery of SD increases in response to the widest AP\(_{I}\) trains (Fig. 6.6B,C, 6.9B). Thus, by increasing [Ca\(^{2+}\)\(_o\)], and thereby decreasing the requirement for Ca\(^{2+}\)-domain overlap in releasing SVs, Pr may be elevated to the point where priming of SVs, without release, is unlikely to occur. Even in immature synapses, where loose VGCC-SV coupling appears more conducive to SV priming when [Ca\(^{2+}\)\(_o\)] is elevated, only use of the narrowest AP\(_{35}\) waveform as a voltage-command resulted in transient I\(_{EPSC}\) facilitation and decreases in SD (Fig. 6.6B, 6.2C). Interestingly, many of the trends observed in SD show analogous dependencies to the facilitation of I\(_{EPSC}\) observed above, supporting the hypothesis that a common mechanism may determine both the direction of SD progression and whether facilitation or depression occurs in I\(_{EPSC}\).

6.2.5 Low Cooperativity Synapses Do Not Exhibit Bidirectionality in Synaptic Delay Independent of Age.

To further explore whether the spatial coupling of VGCCs and SVs influences the direction of SD progression, we performed the above analyses in immature Sept5\(^{-/-}\) synapses, which exhibit a low-\(m\) input-output relationship, and compared them to WT synapses under the same conditions. We hypothesized that immature Sept5\(^{-/-}\) synapses would show SD progression patterns similar to that observed in other low-\(m\) conditions (i.e. mature synapses, immature synapses in 2mM [Ca\(^{2+}\)\(_o\)]).

In response to the wide AP\(_{I}\) trains in 1mM [Ca\(^{2+}\)\(_o\)], immature Sept5\(^{-/-}\) synapses exhibited similar early and late SD progression rates to those of WT synapses of the same developmental stage (\(s_E=1.3\pm 1.8\), \(s_L=1.0\pm 0.6\) vs. \(s_E=0.8\pm 0.5\), \(s_L=1.3\pm 0.3\), Sept5\(^{-/-}\) vs. WT) (Fig. 6.7A, bottom panel, 6.7C). In contrast to the bidirectional progression in SD observed in WT synapses early in trains, Sept5\(^{-/-}\) synapses exhibited only increasing patterns of SD in response to AP\(_{M}\) trains with late SD progression being similar between both genotypes (\(s_E=1.2\pm 1.4\), \(s_L=0.8\pm 0.3\) vs. \(s_E=-2.1\pm 0.7\), \(s_L=0.7\pm 0.1\), Sept5\(^{-/-}\) vs. WT) (Fig. 6.7A, top panel, 6.7B). Interestingly, early SD progression values, measured from immature Sept5\(^{-/-}\) synapses in 1mM [Ca\(^{2+}\)\(_o\)] were similar to those measured in immature WT synapses in 2mM [Ca\(^{2+}\)\(_o\)] (AP\(_{M}\)) or measured in response to the wider AP\(_{I}\) trains in 1mM [Ca\(^{2+}\)\(_o\)] (Fig. 6.9A). Both of these
Figure 6.7: Loss of Septin 5 Reproduces Synaptic Delay Patterns Characteristic of Other Low-Cooperativity Synapses.

(A) Presynaptic $I_{\text{Ca}}$ and postsynaptic $I_{\text{EPSC}}$ from the immature Sept5<sup>−</sup> synapse, in response to AP<sub>H</sub> (top panel) and AP<sub>I</sub> (bottom panel) waveforms, shown in Fig. 6.5B. Three boxed regions, around the first (black), fifth (red), and final (blue) events are magnified and aligned by $I_{\text{Ca}}$ peak at right. Heavy black dashed line denotes time of $I_{\text{Ca}}$ peak and thin dashed lines denote the timing of EPSC onset. (B) Pooled data of SD, normalized to the SD of the first event, against event number for the complete train of events (left panel) and first 20 events (right panel) recorded from immature WT and Sept5<sup>−</sup> synapses in response to the AP<sub>H</sub> waveform. Least-squares linear regression lines are fitted to the first 5 data points and to points 6 to 20 with slope of each segment shown in legend. (C) As in B using the AP<sub>I</sub> waveform.
conditions effectively reduce \( m \) by increasing the \( \text{Ca}^{2+} \) flux per event, thereby minimizing the requirement for \( \text{Ca}^{2+} \)-domain overlap in triggering release of a SV. Likewise, loss of Sept5 appears to tighten VGCC-SV coupling in immature synapses, reducing \( m \), and appears to produce SD progression patterns reminiscent of other low-\( m \) conditions (Fig. 6.4A,B, 6.9A,B). In accordance with a tightening in VGCC-SV coupling, immature Sept5\(^{-/-}\) synapses exhibited raw SD values throughout AP trains that sit between those of WT immature and mature synapses as expected (see Fig. 5.6D).

As an alternate method of reducing \( m \), we loaded immature nerve terminals with 10mM EGTA. When the wide AP\(_{i} \) waveforms were used to evoke \( I_{\text{Ca}} \) and \( I_{\text{EPSC}} \) from EGTA-loaded terminals, raw SD values were significantly lower than those of non-loaded terminals in response to the same APs (data not shown). Both EGTA-loaded and unloaded terminals showed only increases in their SD during the trains with the EGTA-loaded synapses having a significantly greater slope during the early events in response to the AP\(_{i} \) waveform (\( s_{E}=3.2\pm0.5 \) vs. \( 0.8\pm0.5 \)) (Fig. 6.8A, bottom panel, 6.8C). Later in the trains, SD progression equalized between the loaded and unloaded synapses (\( s_{L}=0.9\pm0.2 \) vs. \( 1.3\pm0.3 \)) suggesting that EGTA exerted its effect on SD primarily during the initial events when the wide AP\(_{i} \) trains were used (Fig. 6.8C, 6.9A,B).

In contrast to the transient decreases in SD observed in unloaded synapses early in the AP\(_{M} \) trains, EGTA loaded synapses exhibited only increases in their SD given the same input (\( s_{E}=1.6\pm0.7 \) vs. \( -2.1\pm0.7 \), \( P<0.05 \)) (Fig. 6.8A, top panel, 6.8B). As with the AP\(_{i} \) trains, loaded and unloaded synapses exhibited similar rates of SD increase during the later events of the AP\(_{M} \) trains (\( s_{L}=0.9\pm0.1 \) vs. \( 0.7\pm0.1 \)) (Fig. 6.8B).

The loss of bidirectionality in SD progression, observed in both immature Sept5\(^{-/-}\) and EGTA-loaded synapses, suggests that the synapses ability to transiently increase the rate at which it releases SVs depends on \( P_{r} \) through their coupling to VGCCs. Moreover, decreases in SD appear to occur only when the effective \( m \)-value of the synapse is high (~5-6), this occurring only in immature synapses. In contrast, mature synapses may have progression rates that are very mild, if different from zero at all, however, under no tested conditions does SD shorten at this developmental stage (Fig. 6.9A,B). In immature synapses, independent of the driving force for \( \text{Ca}^{2+} \), the widest AP\(_{i} \) trains never triggered a decrease in SD nor did they ever result in facilitation in \( I_{\text{EPSC}} \). Decreases in SD occurred only during periods of strong \( I_{\text{EPSC}} \) facilitation which occurred either with AP\(_{M} \) trains in 1mM \([\text{Ca}^{2+}]_{o}\) or with AP\(_{35} \) trains in 2mM \([\text{Ca}^{2+}]_{o}\) (Fig. 6.9A).

The above data suggests that there is a relatively narrow range of \( I_{\text{Ca}} \) which can produce transient increases and decreases in \( P_{r} \)STP and SD during repetitive activity. Immature synapses, with their loose VGCC-SV coupling, seem to exhibit this plasticity more robustly than do mature synapses whose synaptic output patterns appear less dependent on changes in \( I_{\text{Ca}} \).

In immature synapses, \( \text{Ca}^{2+} \) influxes of variable magnitudes and durations likely cause different degrees of \( \text{Ca}^{2+} \)-domain overlap which significantly alter the \( P_{r} \) of SVs. Below this particularly dynamic range of \( I_{\text{Ca}} \), \( P_{r} \) is reduced to the point where insignificant SV release occurs (i.e. AP\(_{35} \) in 1mM \([\text{Ca}^{2+}]_{o}\)), while above this range, \( P_{r} \) is elevated to the point where most readily-releasable SVs are released upon
Figure 6.8: Functional Exclusion of Loosely-Coupled VGCC-SV Units Eliminates Bi-Directionality in Synaptic Delay.

(A) Presynaptic $I_{\text{Ca}}$ and postsynaptic $I_{\text{EPSC}}$, from the immature synapse loaded presynaptically with 10mM EGTA shown in Fig. 6.5A, recorded in response to $AP_u$ (top panel) and $AP_i$ (bottom panel) waveforms. Three boxed regions, around the first (black), fifth (red), and final (blue) events are magnified and aligned by $I_{\text{Ca}}$ peak at right. Heavy black dashed line denotes time of $I_{\text{Ca}}$ peak and thin dashed lines denote the timing of EPSC onset.

(B) Pooled data of SD, normalized to the SD of the first event, against event number for the complete train (left panel) and first 20 events (right panel) of events recorded from immature synapses, with and without the presynaptic addition of 10mM EGTA, in response to the $AP_u$ waveform. Least-squares linear regression lines are fitted to the first 5 data points and to points 6 to 20 with slope of each segment shown in legend.

(C) As in B using the $AP_i$ waveform.
Figure 6.9: Early & Late Progression of Synaptic Delay in High & Low Cooperativity Synapses. 
(A) Pooled data showing the early progression rate (first 5 events, %·event⁻¹) for synaptic delay in response to AP₃₅, APₙ, and APᵢ waveforms, for all experimental conditions in which reliable synaptic transmission was recorded. (B) As in A showing pooled data for the late progression rate (events 6 through 20, %·event⁻¹) of synaptic delay. See Table 6.1 for tests of significance (Student’s t-test) between all experimental groups in panels A and B.
AP innervation and thus would not be available for priming (i.e. AP$_1$ in 2mM [Ca$^{2+}$]$_o$). These large Ca$^{2+}$ influxes result in strong synaptic depression, and increases in SD throughout the train, while reducing $m$ towards it’s lower limit with larger and larger $I_{Ca}$ (Gentile & Stanley, 2005; Fedchyshyn & Wang, 2005).

Late in the AP trains, where $I_{EPSC}$ depression predominates, SD generally progresses at approximately similar rates showing little dependence on the experimental condition in mature synapses. In contrast, immature synapses show a stronger dependence of SD on AP-width late in trains (Fig. 6.9B). This dependence is strongest in 2mM [Ca$^{2+}$]$_o$, however, it should be noted that the range of late SD progression values is much smaller than those observed in early SD progression (scale differences between Fig. 6.9A,B). This implies that the mechanism underlying the late phase of SD prolongation is relatively insensitive to the experimental conditions but seems to depend somewhat on the developmental stage of the synapse.

As hypothesized previously, depletion of the pool of available SVs could result in a global increase in SD while replacement SVs are transiting to release sites and becoming fusion-competent (Wu & Borst, 1999; Sakaba & Neher, 2001). The rate of depletion of the RRP would then depend both on the size of the RRP and on the $P_a$, which in turn depends both on the magnitude of Ca$^{2+}$ influx and the coupling of VGCCs and SVs (Augustine et al., 1985; Sabatini & Regehr, 1997; Sakaba & Neher, 2001; Fedchyshyn & Wang, 2005). Given the larger size of the RRP in mature synapses, we would expect depletion to have less of an effect on SD in these synapses than in immature synapses, which is in accordance with the data above (Taschenberger & von Gersdorff, 2000). Alternatively, activity-dependent increases in SD could be the result of changes to a number of other processes in the SV release cascade including; activity-dependent inactivation of $I_{Ca}$, a change in the functional state of the release machinery, or Ca$^{2+}$-dependent exhaustion/desensitization of the release machinery (Betz, 1970; Hsu et al., 1996; Forsythe et al., 1998). In any of these alternative mechanisms, increased Ca$^{2+}$ influx, with increasing stimulus number, could result in increased SD and synaptic depression. Given these possibilities, we sought to determine whether increases in SD were the result of SV depletion or a mechanism upstream.

6.2.6 Increases in SD Are Due to Activity-Dependent Depletion of the Readily Releasable Pool of Synaptic Vesicles.

To address whether increases in SD occur as the result of depletion of the RRP or due to Ca$^{2+}$-dependent exhaustion/desensitization of the release machinery, we employed a presynaptic voltage-command protocol that would increase intra-terminal [Ca$^{2+}$]$_i$ ([Ca$^{2+}$]$_i$) without triggering significant SV release. The protocol consisted of two AP-like waveforms, similar to AP$_M$ APs in their width and amplitude (triangle wave, 40ms half-width, 120mV), separated by a mild depolarizing step of 20mV (-80 to -60mV) of either 20ms, 200ms, or no step at all (control) (Fig. 6.10A). All recordings were conducted in 1mM [Ca$^{2+}$]$_o$. Step durations were chosen to correlate with the length of time required to achieve the maximum reduction or increase in SD observed in response to the various AP trains (i.e. event 5 and event 40).
Figure 6.10: Shortening of Synaptic Delay Depends on Increases in Residual \([\text{Ca}^{2+}]\).

(A) Presynaptic voltage command waveform used to determine the dependence of SD on residual \([\text{Ca}^{2+}]\). Intermediate step to 60mV is set to either 20ms or 200ms duration as noted. (B) Presynaptic \(I_{\text{ca}}\) and postsynaptic \(I_{\text{EPSC}}\), recorded from an immature (top panel) and mature (bottom panel) synapse, with (top sub-panel) and without (bottom sub-panel) the 20ms intermediate step to -60mV. Events in black are in response to the first AP-like waveform with events in red being in response to the second AP-like waveform. Boxed regions are magnified, overlaid, and aligned by \(I_{\text{ca}}\) peak on right. (C) Presynaptic \(I_{\text{ca}}\) and postsynaptic \(I_{\text{EPSC}}\), recorded from an immature (top panel) and mature (bottom panel) synapse, with (top sub-panel) and without (bottom sub-panel) the 200ms intermediate step to 60mV. Events in black are in response to the first AP-like waveform with events in red being in response to the second AP-like waveform. Boxed regions are magnified, overlaid, and aligned by \(I_{\text{ca}}\) peak on right.
A prolonged step of as little as 10mV has been shown to result in an optically measurable increase in intra-terminal [Ca$^{2+}$], even though the same increase in [Ca$^{2+}$] could not be detected in presynaptic electrophysiological recordings of $I_{Ca}$, and result in Ca$^{2+}$-dependent potentiation of $I_{EPSC}$ (Awatramani et al., 2005). Using a mild depolarizing step of this type allows for Ca$^{2+}$ ions to enter the terminal, at levels which may be sufficient to activate processes upstream of SV release and which may trigger the mechanisms, independent of SV depletion, which could underlie increases in SD. Therefore, if an increase in SD is observed in response to the second AP, then depletion of the RRP can be excluded as the mechanism for prolongation in SD. Alternatively, if SD is stable, despite the increase in [Ca$^{2+}$], then Ca$^{2+}$-dependent processes, independent of RRP depletion, can be mechanistically excluded. Furthermore, this protocol may provide further evidence as to whether elevations in [Ca$^{2+}$], which prime SV for subsequent release, underlie the transient decreases in SD observed under conditions of high-$m$.

During the 20mV steps, our recordings did not register changes in $I_{Ca}$ discernible from ambient recording noise; however, increasing the step amplitude to approximately 25mV or larger resulted in measurable $I_{Ca}$ during the same period (data not shown). Particularly in mature synapses, these larger amplitude steps were accompanied by significant SV release which, if present, could result in moderate depletion of the RRP and complicate interpretation of the results. For this reason, intermediate steps were kept to 20mV (-80 to -60mV). The AP-like waveforms triggered similar $I_{Ca}$ from both immature and mature synapses but resulted in significantly smaller $I_{EPSC}$ in immature synapses as expected (Fedchyshyn & Wang, 2005) (Fig. 6.10B,C). For short intervals, both immature and mature synapses exhibited $I_{Ca}$ facilitation in response to the second AP whether the 20ms step was applied or not (Fig. 6.10B, 6.11A). Immature synapses showed 7.8±0.8% and 4.9±0.6% $I_{Ca}$ facilitation while mature synapses showed increases of 6.9±0.1% and 6.0±0.6% with and without the 20ms step respectively (Fig. 6.10B, 6.11A). Facilitation in $I_{Ca}$ was accompanied by facilitation in $I_{EPSC}$ of 56±13% and 28±12% for immature synapses and 22±9% and 15±5% for mature synapses with and without the 20ms step respectively (Fig. 6.10B, 6.11C). Immature synapses showed significantly greater $I_{EPSC}$ facilitation following the 20ms steps than did mature synapses, while no significant difference existed across development in the absence of the steps (Fig. 6.11C).

In response to the longer 200ms steps, both immature and mature synapses exhibited $I_{Ca}$ facilitation of 2.7±0.5% and 4.8±0.9% respectively, however in the absence of the step, no significant $I_{Ca}$ facilitation was observed in either experimental group (Immature: 0.4±0.5%, Mature: 0.2±0.6, p>0.05) (Fig. 6.10C, 6.11A). Likewise, significant $I_{EPSC}$ facilitation occurred only when the 200ms step was applied, with immature $I_{EPSC}$ increasing by 27±5% and mature $I_{EPSC}$ increasing by 20±6% (Fig. 6.10C, top panel, 6.11C). Omission of the 200ms step resulted in $I_{EPSC}$ that were not significantly different from 0 for either immature (11±7%) or mature (-7±4%) synapses, but were significantly different from each other (Fig. 6.12C).

In immature synapses, $I_{Ca}$ facilitation depended partially on the duration between APs, with shorter durations resulting in significantly larger increases in $I_{Ca}$, and partially on the presence of a step,
Figure 6.11: Neither Depression of $I_{\text{EPSC}}$ or Prolongation in Synaptic Delay Occurs in the Absence of Synaptic Vesicle Depletion.

(A) Pooled data plotting the percent change in $I_{\text{Ca}}$, with and without the 20/200ms step, for immature and mature synapses. (B) Pooled data plotting the percent change in $I_{\text{EPSC}}$, with and without the 20/200ms step, for immature and mature synapses. (C) Pooled data plotting the percent change in SD, with and without the 20/200ms step, for immature and mature synapses. All experiments were performed in 1mm $[\text{Ca}^{2+}]_o$. Single asterisks (*) denote statistically significant difference between data sets shown, double asterisks (**) denote data not statistically different from 0 (Student's t-test, p<0.05).
which also produced larger increases in $I_{Ca}$ than in its absence ($p<0.05$) (Fig. 6.11A). These data confirm that the degree of $I_{Ca}$ facilitation depends on both the inter-stimulus-interval and on the build-up of $[Ca^{2+}]_o$, as previously reported at this synapse (Cuttle et al., 1998). With long durations (i.e. 200ms), we observe only the portion of $I_{Ca}$ facilitation dependent on $Ca^{2+}$, as it is absent when the step is omitted (Fig. 6.10B, bottom panel, 6.11A). Mature synapses exhibit no difference in $I_{Ca}$ facilitation, with and without 20ms steps, suggesting a weak $Ca^{2+}$ dependence. However, for long durations, the presence of $I_{Ca}$ facilitation depended completely on whether a step was applied (Fig. 6.10C, bottom panel, 6.11A). These data suggest that multiple factors determine whether, and to what degree, $I_{Ca}$ facilitation occurs in both the immature and mature synapses.

Synaptic output generally followed from $I_{Ca}$ in immature synapses, being largest for short intervals and in the presence of a step, and being significantly larger than that of mature synapses under the same conditions. No detectable $I_{EPSC}$ facilitation was observed in the absence of the 200ms steps, as would be expected from the stability of $I_{Ca}$ under these conditions. This was true of both immature and mature synapses (Fig. 6.10B,C, 6.11C).

From the data above, we also assayed the impact of elevating $[Ca^{2+}]_o$ on SD. For short intervals, immature synapses exhibited significant decreases in SD following the 20ms step, with an average $-5\pm1\%$ decrease (Fig. 6.10B, 6.11B). In the absence of the step, the same immature synapses showed no significant change in their SD between APs ($2\pm2\%$). When the inter-event-interval was lengthened, immature synapses showed a similar trend in SD, decreasing when the 200ms step was applied ($-5\pm2\%$) and showing no change when the step was omitted ($0\pm2\%$) (Fig. 6.10C, 6.11B). In contrast, independent of the presence of a step, or the duration of the inter-event-interval, mature synapses showed no significant change in their SD (Fig. 6.10B,C, 6.11B). From these observations it appears that, despite lengthy sub-threshold influxes of $Ca^{2+}$ during the step, neither immature or mature synapses showed an increase in SD on AP$_2$, suggesting that $[Ca^{2+}]_o$ itself is not acting to prolong release kinetics. More likely is that depletion of SVs from the RRP underlies activity-dependent increases in SD.

Interestingly, only immature synapses show decreases in SD between AP$_1$ and AP$_2$ and only when an intermediate step is present (Fig. 6.11B), however, despite significant $I_{Ca}$ facilitation, no decrease in SD is observed from immature synapses in the absence of the 20ms step. This suggests that priming of the $Ca^{2+}$ sensor for release (i.e. synaptotagmin), and the accompanying increase in P$_n$, most likely underlies these observations in immature synapses. However, the fact that $I_{Ca}$ facilitation occurs in response to both conditions in which SD decreases (i.e. with either duration of step), complicates interpretation of the results. We have shown previously that SD is inversely related to the magnitude of $I_{Ca}$, a dependence that is much steeper in immature synapses than in mature ones (see section 5.2.2). Thus it is conceivable that the $I_{Ca}$ facilitation observed here could underlie the shortening in SD observed in immature synapses and/or potentially mask $Ca^{2+}$-dependent SD lengthening in both immature and mature synapses. There also exists some question, in the absence of a direct read-out, as to whether $Ca^{2+}$ actually enters the nerve terminal during the steps, despite evidence from optical measurements under similar experimental paradigms (Awatramani et al., 2005). To address the latter
Figure 6.12: 20mV Depolarizations Evoke Significant Increases in mEPSC Frequency.

(A) Presynaptic voltage command waveform used to evoke the traces shown in B. (B) 50 superimposed traces of mEPSCs from an immature (top panel) and mature (bottom panel) synapse in response to the presynaptic voltage command waveform in A. Dashed lines denote the beginning and end of the depolarizing step. (C) Pooled data showing mEPSC frequency, during and outside the 200ms depolarization to 60mV, in immature synapses (n=6). (D) As in C for mature synapses (n=10). (E) Pooled data showing the average number of quanta released, per sweep, during a 200ms step depolarization to 60mV in immature and mature synapses. Asterisks denote statistical significance (Student's t-test, p<0.05).
question, we performed high-gain whole-cell recordings of postsynaptic miniature EPSCs (mEPSCs) evoked during, and outside, the 200ms presynaptic voltage steps from –80mV to –60mV (Fig. 6.12A). Outside the 200ms step, immature synapses showed a characteristically lower mEPSC frequency than mature synapses (0.2±0.02Hz vs. 9±2Hz) (Joshi et al., 2004) (Fig. 6.12B,C,D). Both of these frequencies were slightly higher than those generally observed in the absence of presynaptic whole-cell invasion of the nerve terminal, the difference most likely caused by insult to the terminal during the recording process. During the 200ms step, mEPSC frequency increased significantly to 0.4±0.07Hz and 21±3Hz in immature and mature synapses respectively (Fig. 6.12B,C,D). The increase in mEPSC frequency, observed in both developmental stages during the step, suggests that the mild 20mV depolarization, used to trickle Ca\(^{2+}\) into the terminal, provides enough Ca\(^{2+}\) influx to significantly increase the rate of SV release. However, as discussed previously, in order to confidently determine whether increases in SD are the result of depletion of the RRP, the number of SVs released during the intermediate steps (i.e. Fig. 6.10) must be insignificant compared to the total size of the RRP.

In immature synapses, the average number of quanta released during a 200ms step was 0.07±0.01 (Fig. 6.12E), which is approximately 0.002% to 0.008% of the RRP, based on a range of estimates for the total RRP of between 900 and 4000 (Scheuss et al., 2002; Neher & Sakaba, 2001; Sun & Wu, 2001; Wölfel & Schneggenberger, 2003). These values should be approximately 10-fold lower for the shorter 20ms steps. For mature synapses, in which tight VGCC-SV coupling results in a much larger excretion of SVs during the steps, the average number of quanta released was 4.2±0.6 (Fig. 6.12E). While few estimates for the RRP size exist for this developmental stage, it has been estimated that the RRP size of immature synapses (P5-7) is approximately 30% of that measured from juvenile synapses (P12-14) (Taschenberger et al., 2002). Using this measure, suggesting an RRP of 1500-6600, mature synapses release 0.06-0.3% of their RRP during a 200ms step to –60mV. Thus, the steps used are very unlikely to deplete the RRP to the extent required to evoke prolongation in release kinetics. In addition, the elevated mEPSC frequency, most likely a result of the recording conditions, probably results in an overestimation of these values, confirming the above interpretation.

### 6.2.7 Shortening in Synaptic Delay Occurs Independent of Ca\(^{2+}\)-Current Facilitation in Immature Synapses.

As discussed above, reductions in SD can occur as a result of transient increases in P_r, occurring when \([\text{Ca}^{2+}]_o\) is elevated in the nerve terminal, and in response to increases in the magnitude of I_{Ca} (see section 6.2.6). To determine whether increases in P_r, independent of facilitation in I_{Ca}, could result in a decrease in SD, we repeated the experiments shown in Figure 6.10 but modulated the amplitude of the AP_{2} to compensate for facilitation in I_{Ca} (Fig. 6.13A). During each experiment, the peak amplitude of AP_{2} was adjusted to approximately 35-38mV from 40mV, which varied between cells, in order to equalize I_{Ca} from both APs. For experiments in which I_{Ca} facilitation did not occur (i.e. in the absence of 200ms steps), no adjustment of the second AP was necessary. In all experiments, changes in I_{Ca} were kept to less than ±2% with most experiments having changes in I_{Ca} controlled to less than ±1% (Fig. 6.13B,C, Fig. 6.14A). Under no conditions were changes in I_{Ca} statistically different from 0...
Figure 6.13: Ca\(^{2+}\)-Dependent Shortening in Synaptic Delay is Independent of I\(_{\text{Ca}}\) Facilitation.

(A) Presynaptic voltage command waveform used to determine the dependence of SD on residual [Ca\(^{2+}\)]. Intermediate step to -60mV is set to either 20ms or 200ms duration as noted. Note that the amplitude of the second AP-like waveform (red) is adjusted to generate I\(_{\text{Ca}}\) of the same amplitude as that in response to the first (black) AP-like waveform. **(B)** Presynaptic I\(_{\text{Ca}}\) and postsynaptic I\(_{\text{EPSC}}\), recorded from an immature (top panel) and mature (bottom panel) synapse, with (top sub-panel) and without (bottom sub-panel) the 20ms intermediate step to -60mV. Events in black are in response to the first AP-like waveform with events in red being in response to the second AP-like waveform. Boxed regions are magnified, overlaid, and aligned by I\(_{\text{Ca}}\) peak on right. **(C)** Presynaptic I\(_{\text{Ca}}\) and postsynaptic I\(_{\text{EPSC}}\), recorded from an immature (top panel) and mature (bottom panel) synapse, with (top sub-panel) and without (bottom sub-panel) the 200ms intermediate step to -60mV. Events in black are in response to the first AP-like waveform with events in red being in response to the second AP-like waveform. Boxed regions are magnified, overlaid, and aligned by I\(_{\text{Ca}}\) peak on right.
(Student’s t-test, p>0.05) (Fig. 6.14A). With changes in $I_{\text{Ca}}$ well controlled, we could better determine whether transient increases in $P_r$, in response to increases in [Ca$^{2+}$], were sufficient to decrease SD. Furthermore, we can eliminate developmental differences in $I_{\text{Ca}}$ facilitation as a confounding factor in determining whether differences in VGCC-SV coupling, between immature and mature synapse, underlie a propensity towards bidirectionality in SD.

In the absence of $I_{\text{Ca}}$ facilitation, mature synapses showed little change in their $I_{\text{EPSC}}$ amplitude. For short durations, mature synapses showed $-2\pm2\%$ and $-14\pm3\%$ changes in $I_{\text{EPSC}}$, with and without a 20ms step respectively (Fig. 6.13B, 6.14B). This trend was similar for longer durations with changes in $I_{\text{EPSC}}$ of $-5\pm2\%$ and $-8\pm2\%$ with and without 200ms steps respectively (Fig. 6.13C, 6.14B). In the presence of either duration of step, changes in $I_{\text{EPSC}}$ were not significantly different from 0 (p>0.05), while in the absence of either step, $I_{\text{EPSC}}$ actually showed slight but significantly (Fig. 6.14B). These results suggest that, despite an increase in [Ca$^{2+}$], generated by the steps, mature synapses do not exhibit increases in $P_r$ sufficient to trigger facilitation in $I_{\text{EPSC}}$. While $I_{\text{EPSC}}$ facilitation does not occur in mature synapses in the absence of $I_{\text{Ca}}$ facilitation, a slight increase in $P_r$ may occur and act to attenuate depression somewhat following the steps (Fig. 6.14B). Loss of $I_{\text{Ca}}$ facilitation did not impact SD in mature synapses as no significant changes were observed between the APs for either short (with 20ms step: $2\pm1\%$; without 20ms step: $-1\pm1\%$) or long intervals (with 200ms step: $-1\pm2\%$; without 200ms step: $3\pm2\%$) (Fig. 6.14C).

Even in the absence of $I_{\text{Ca}}$ facilitation, immature synapses showed robust facilitation in $I_{\text{EPSC}}$ for all but long intervals in the absence of a step (Fig. 6.13B, C, 6.14B). In response to a 20ms step, $I_{\text{EPSC}}$ increased $36\pm8\%$, while in the absence of the same step, $I_{\text{EPSC}}$ increased $19\pm6\%$ (Fig. 6.14B). $I_{\text{EPSC}}$ increased by $38\pm11\%$, following 200ms steps, while no change in $I_{\text{EPSC}}$ was observed in the absence of the same step ($-7\pm6\%$, p>0.05) (Fig. 6.13C, 6.14B). Immature synapses continued to exhibit decreased SD in response to either 20ms or 200ms steps (20ms: $-5\pm2\%$, 200ms: $-3\pm2\%$) but to a lesser extent than when $I_{\text{Ca}}$ facilitation was present (Fig. 6.14C, 6.11B). In the absence of either duration of step, no significant difference in SD was observed (20ms: $-2\pm2\%$, 200ms: $1\pm3\%$), as was the case in the presence of $I_{\text{Ca}}$ facilitation (Fig. 6.14C, 6.11B).

These data show that, unlike in mature synapses, both the kinetics and magnitude of release events in immature synapses can be transiently increased solely through increasing [Ca$^{2+}$]. The fact that $P_r$ is also enhanced for short intervals, in the absence of a step, suggests that even a small increase in [Ca$^{2+}$], generated from the first AP, may be sufficient to potentiate SV release for subsequent APs. In immature synapses, $I_{\text{Ca}}$ facilitation and elevations in [Ca$^{2+}$] contribute additively to increase synaptic output. In contrast, in mature synapses $I_{\text{Ca}}$ facilitation appears to be the prevailing presynaptic mechanism potentiating SV release. Interestingly, only in immature synapses, does the combination of $I_{\text{Ca}}$ facilitation and Ca$^{2+}$-dependent increases in $P_r$ manifest as a measurable decrease in SD. Finally, since no significant prolongations in SD were observed in either developmental group, under any of the experimental conditions, we conclude that RRP depletion is the most likely mechanism accounting for activity-dependent increases in SD in both immature and mature synapses.
These data suggest that mature synapses, by virtue of their tight VGCC-SV coupling, exhibit significantly less plasticity in their synaptic output, in both SV release kinetics and strength, than do immature synapses. Loose coupling of VGCCs and SVs allows for priming of SVs, without release, which is integral for the transient increase of $P_r$ during activity, and the expansion of the immature synapses dynamic range during this equally dynamic period of development. Thus, the developmental switch from microdomain to nanodomain release modalities is likely a key presynaptic adaptation which aids in reducing dynamic range and consolidating this, and potentially other, central synapses’ ability to reproduce high-frequency signals faithfully.
Figure 6.14: \( I_{\text{EPSC}} \) Facilitation & Decreases in Synaptic Delay Occur Only in Immature Synapses in the Absence of \( I_{\text{Ca}} \) Facilitation.

(A) Pooled data plotting the percent change in \( I_{\text{Ca}} \), with and without the 20/200ms step, for immature and mature synapses.  (B) Pooled data plotting the percent change in \( I_{\text{EPSC}} \), with and without the 20/200ms step, for immature and mature synapses.  (C) Pooled data plotting the percent change in SD, with and without the 20/200ms step, for immature and mature synapses.  All experiments performed in 1mM \([\text{Ca}^{2+}]_o\).  Single asterisks (*) denote statistically significant difference between data sets shown, double asterisks (**) denote data not statistically different from 0 (Student's t-test, \( p<0.05 \)).
6.3 DISCUSSION:

Short-term plasticity (STP) has many underlying mechanisms in different synapses and has diverse implications for signal processing and filtering in the central nervous system (Atwood & Wojtowicz, 1986; Zucker, 1993; Fortune & Rose, 2002; von Gersdorff & Borst, 2002; Zucker & Regehr, 2002; Abbott & Regehr, 2004). Ultimately, plasticity, whether potentiating (i.e. STF) or attenuating (i.e. STD), arises from an accompanying increase or decrease in release probability ($P_B$), which may be due to any combination of mechanisms (Zucker & Regehr, 2002). Many of these mechanisms are dynamic during repetitive activity and can also depend on previous activity, largely through resulting changes to the intra-terminal $[Ca^{2+}]_{(P)}$ (Dittman et al., 2000; Zucker & Regehr, 2002; von Gersdorff & Borst, 2002).

Further complicating elucidation of the mechanisms underlying STP are observations that influencing factors such as; AP width, VGCC localization, number of AZs, RRP size, synaptic morphology, and $Ca^{2+}$ buffering, do or may change with synaptic development to affect $P_B$ (Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; Fedchyshyn & Wang, 2005; Yang & Wang, 2006).

Here we asked three key questions concerning STP at the calyx of Held-MNTB synapse; (1) We asked whether the different spatial coupling between VGCCs and SVs observed in immature and mature synapses determines the patterns of STP observed across development. (2) We asked whether the changes in $P_B$ that underlie the alterations in synaptic strength ($I_{EPSC}$) during STP, also impact the kinetics of release (SD). (3) Finally, we asked what mechanisms underlie the STF/STD patterns observed across development at this synapse.

Our results confirm that the nature of VGCC-SV coupling, or release modality, provides a platform through which certain forms of STP, in both synaptic strength and timing, can be expressed across development. This arises due to a shift in the key upstream probabilistic factors on which release of a SV depends, from the coincident opening of many VGCCs in immature synapses, to the individual $P_o$ of the VGCCs in mature ones. Finally, we show that $Ca^{2+}$-dependent priming of the release machinery is crucial for the expression of STF and decreases in SD, occurring most readily in immature synapses, and that SV depletion underlies STD and activity-dependent increases in SD at both developmental stages.

Our proposed model considers the $P_r$ of an individual SV as depending on two factors. First, if VGCC(s) open in the vicinity of an SV and second, given that opening, we examined whether the local $Ca^{2+}$ signal is sufficient to satisfy the binding criterion of the $Ca^{2+}$-sensor. Placing this model in the context of the developing calyx of Held-MNTB synapse, it is clear that the change from loose to tight VGCC-SV coupling must significantly alter the strategies employed by the synapse in releasing SVs. In immature, loosely-coupled, synapses, satisfaction of the $Ca^{2+}$-sensor binding criterion depends largely on the coincident opening of many VGCCs in the vicinity of a SV supplying a suprathreshold $I_{Ca}$ signal - a highly non-linear process (Borst & Sakmann, 1996; Wu et al., 1999; Fedchyshyn & Wang, 2005). At these synapses, wide APs ensure sufficient VGCC openings and $I_{Ca}$ to generate reliable SV release (Taschenberger & von Gersdorff, 2000) while narrowed APs produce severely attenuated or no SV release at all (Fedchyshyn & Wang, 2005). In contrast, at mature tightly-coupled synapses,
suprathreshold Ca\(^{2+}\) signals are likely to be produced at the Ca\(^{2+}\)-sensor upon one or few VGCC openings, coupling SV release closely with the actual activation of the VGCCs by the AP, a more linear process (Augustine, 1990; Fedchyshyn & Wang, 2005). At these synapses, narrow APs restrict the number of VGCCs opened and thereby constrain SV release to levels similar, or slightly larger, than those observed in immature synapses (Chuma & Ohmori, 1998; Taschenberger & von Gersdorff, 2000; Futai et al., 2001; Iwasaki & Takahashi, 2001; Joshi & Wang, 2002; Yang & Wang, 2006). This balance most likely serves to maintain synaptic efficacy while avoiding overly rapid depletion of SVs during prolonged bouts of synaptic activity (Taschenberger & von Gersdorff, 2000; Yang & Wang, 2006). However, the question remains as to how this change in release strategy alters the effectiveness of transmission and characteristics of STP observed at the synapse?

We addressed this question by using trains of recorded APs (200Hz, 200ms), of various widths, as presynaptic voltage-clamp commands to generate I\(_{Ca}\), and the corresponding I\(_{EPSC}\), from immature and mature synapses. Using this experimental paradigm we were able to confine our analysis, regarding the impact of VGCC-SV coupling on STP, to factors downstream of I\(_{Ca}\), thereby eliminating changes in AP width as a confounding factor in our analysis. Interestingly, when we applied these trains in both immature and mature synapses, we observed that immature synapses are much more likely to show STF than are mature synapses, which tend to exhibit only STD for a given AP waveform (Fig. 6.1, 6.2). In response to this observation, we asked how this finding could be rationalized based on developmental differences in transmission downstream of I\(_{Ca}\).

SVs, in immature synapses, appear to be arranged such that they exhibit a heterogeneous distribution of P, which may arise due to heterogeneous VGCC-SV separation distances (Meinrenken et al., 2002, Meinrenken et al., 2003, Trommershäuser et al., 2003; Fedchyshyn & Wang, 2005; Wadel et al., 2007). Evidence to support this hypothesis arises from the observation that when EGTA is loaded into immature nerve terminals, a reduced but significant population of SVs are released in response to I\(_{Ca}\) generated by AP-like presynaptic voltage-commands. This suggests that there is a distribution of VGCC-SV units, some of which are loosely-coupled (EGTA-sensitive), and some of which that are more tightly-coupled (EGTA-insensitive) (Borst & Sakmann, 1996; Fedchyshyn & Wang, 2005). In addition, some SV units are distant enough from VGCCs that they cannot be released by AP-generated I\(_{Ca}\) and require flash photolysis of caged-Ca\(^{2+}\) or significant enhancement of I\(_{Ca}\) to trigger their release (Wadel et al., 2007; see also Moulder & Mennerick, 2005). Thus, in response to an AP, loosely-coupled SVs may experience subthreshold Ca\(^{2+}\) signals which fail to elevate [Ca\(^{2+}\)], at the Ca\(^{2+}\)-sensor, to suprathreshold levels. However, this subthreshold Ca\(^{2+}\) signal builds [Ca\(^{2+}\)], in the vicinity of the SV and may serve to prime it for subsequent AP innervations. Interestingly, when the structure of the tightly-coupled AZs of the frog NMJ were disrupted with low [Ca\(^{2+}\)]\(_{o}\) treatment, presumably uncoupling VGCCs from release proteins, increased PPF was observed along with a decrease in AP-evoked SV release (Meriney et al., 1996). This finding suggests that the spatial relationship between VGCCs and SVs can impact the nature of STP observed.

Our formulation of the “residual Ca\(^{2+}\) hypothesis” (Katz & Miledi, 1968; Katz, 1969) requires that a SV be primed but not released in order for it to contribute to STF. Thus, the mixed-modality VGCC-SV
units, present in immature synapses, can establish a population of SVs that are likely to experience a range of supra- and subthreshold Ca\(^{2+}\) signals, heterogeneity in P_s, and the resulting STF. By shaping the size and spread of Ca\(^{2+}\)-domains, the efficacy and saturation level of endogenous buffers are likely to underlie attenuation of the Ca\(^{2+}\) signal experienced at distant SVs, selectively and specifically promoting STF at them (Roberts, 1994; Klingauf & Neher, 1997; Naraghi & Neher, 1997; Felmy et al., 2003; Wadel et al., 2007). By either broadening the AP or increasing the driving force for Ca\(^{2+}\), we have respectively increased the number of VGCCs contributing to I_{Ca} or increase their single-channel conductance, thereby increasing the likelihood (P_r) that a SV will be released at a particular site (Fig. 6.1, 6.2). While either of these manipulations will increase the number of SVs released in response to a given input, they likewise decrease the number of SVs that remain to be primed, thus decreasing STF.

In the sense that the above is a “positional” model of Ca\(^{2+}\)-secretion coupling, it does not explicitly require the presence of a second high-affinity Ca\(^{2+}\) sensor (Yamada & Zucker, 1992; Bertram et al., 1996; Dittman et al., 2000; Tang et al., 2000), nor does it require that Ca\(^{2+}\) signals from neighbouring AZs interact to prime each other and result in STF (Zucker & Regehr, 2002), but does not exclude these as contributing factors either. It only requires that elevations in [Ca\(^{2+}\)]_i result in Ca\(^{2+}\) ions binding to a Ca\(^{2+}\)-sensor without triggering release, thereby transiently increasing their P_r. In addition, this model focuses exclusively on the presynaptic elements of STP and does not explicitly consider potential postsynaptic mechanisms. However, the effect of either AMPAR saturation or desensitization on our readout of STF (I_{EPSC}), is likely to result in an underestimation of facilitation, especially in immature terminals where these mechanisms of STD are more efficacious (Otis et al., 1996; Neher & Sakaba, 2001; Sheuss et al., 2002; Felmy et al., 2003; Yamashita et al., 2003; Joshi et al., 2004; Koike-Tani et al., 2005).

The absence of STF in mature synapses, in response to all but the narrowest APs (AP_{35}), suggests that very few SVs are primed following the initial AP(s). This suggests that the distribution of VGCCs and SVs may be relatively homogeneous, analogous to the fast pool of SVs modeled by Trommershäuser et al., 2003 to have tightly-coupled VGCCs enhancing their P_r (see also Wadel et al., 2007). Because heterogeneity in P_r depends largely on non-linearities upstream of SV release, the low Ca\(^{2+}\)-domain cooperativity (m) observed in mature synapses (~2-3) causes the range of P_r expected across sites to becomes more heavily dependent on the effectiveness of the AP in opening VGCCs, which is linear (Augustine, 1990). In contrast, loosely-coupled VGCC-SV units add a high degree of non-linearity, through high Ca\(^{2+}\)-domain cooperativity (~5-6), which amplifies input variability into extensive heterogeneity in P_r. Unfortunately, little modeling work has been performed in specific reference to the mature calyx of Held, due to inherent experimental difficulties and the resulting lack of age-specific model parameters. Future, experimental work should at least attempt to extract the necessary model parameters from mature synapses so that the generally accepted model requirement of heterogeneity in P_r, for immature synapses, can be tested later in development. In support of a more homogeneous population of SVs in mature synapses, recent evidence has shown an increase in the amount of synchronous release, at the expense of asynchronous release, observed from the mature calyx of Held (Taschenberger et al., 2005).
In addition to a propensity for STF, we observed that immature synapses exhibited bi-directionality in their progression of SD, which approximately correlated with similar bidirectional changes in \( P_r \) and \( \Delta EPSC \) (i.e. STF/STD). This was observed only under conditions where \( I_{Ca} \) was moderate such as in 1mM \([Ca^{2+}]_o\) with \( AP_M \) or 2mM \([Ca^{2+}]_o\) with \( AP_{35} \), as was the case for STF in \( \Delta EPSC \) in the same synapses (Fig. 6.1,6.2). In contrast, mature synapses never exhibited such bidirectionality in SD under any experimental conditions. Interestingly, immature low-\( m \) synapses (\( Sept5^{-/-} \) and EGTA loaded) displayed only unidirectional SD progression.

These observations raised the possibility that the different ranges of \( P_r \), enabled by the different coupling modalities employed throughout development, could not only produce distinct patterns of STP, but could also determine SD. Based on early modelling studies, it was predicted that STF should be accompanied by a decrease in the latency of transmission (Yamada & Zucker, 1992; Winslow et al., 1994; Bertram et al., 1996), however, it was also predicted, and experimental evidence confirmed, that these changes would prove too small to measure (Daytner & Gage, 1980; Parnas et al., 1989). Alternatively, the methods used to calculate SD may have been of insufficient temporal resolution to resolve small changes on the order of those observed here (see Fedchyshyn & Wang, 2007). More recently, a number of studies have shown that SD can be measurably decreased when \( P_r \) and \([Ca^{2+}]_i\) is rapidly elevated, either through the use of long voltage steps or through flash photolysis of caged \( Ca^{2+} \) compounds (Vyshedsiky & Lin, 1997; Borst & Sakmann, 1999; Vyshedsiky et al., 2000; Bollmann & Sakmann, 2005). Conversely, increases in SD have been associated with the decrease in \( P_r \) observed during extreme synaptic depression (Wu & Borst, 1999; Waldeck et al., 2000; Sun & Wu, 2001; Sakaba & Neher, 2001; Hermann et al., 2007) and more recently, in response to physiological trains of stimuli through increases in \([Ca^{2+}]_i\) (Fedchyshyn & Wang, 2007). We hypothesize, in agreement with others (Wu & Borst, 1999; Sakaba & Neher, 2001; Sun & Wu, 2001), that the increase in SD results from a depletion of the pool of available SVs and that recruitment of those SVs distant from VGCCs underlies the increased delay (Fedchyshyn & Wang, 2007). In accordance, we observe that SD progression rates are highest for the wide AP1 waveforms and milder for the narrow AP35 APs, in both immature and mature synapses, correlating SD prolongation with the extent and rate of SV depletion (Fig. 6.5, 6.6).

Alternatively, a form of \( Ca^{2+} \)-dependent exhaustion of the release machinery, desensitization of the \( Ca^{2+} \)-sensor, or sequential use of SVs with differing intrinsic \( Ca^{2+} \) sensitivities has been suggested to account for the delayed phasic release (Wu & Borst, 1999; Waldeck et al., 2000; also see Lin & Faber, 2002 for review; Wölfel et al., 2007). However, these possibilities seem unlikely given recent findings from the calyx of Held showing that the sensitivity of the immature release apparatus is maintained even when the synapse is depressed (Wadel et al., 2007). Moreover, evidence from a variety of preparations suggests that local \([Ca^{2+}] \) and the nature of the \( I_{Ca} \) ultimately determines the kinetics of release through changes in \( P_r \) (Llinas et al., 1981; Augustine & Charlton, 1986; Borst & Sakmann, 1998; Bollmann et al., 2000; Schneggenburger & Neher, 2000; Meinrenken et al., 2002; Wadel et al., 2007).

From our data, two additional lines of evidence suggest that increases in SD are the result of SV depletion; first, the presynaptic addition of EGTA, which effectively reduces the cooperativity (\( m \)) of immature synapses, decreased the amount of STF for a given AP waveform, eliminated bidirectionality in
SD, and reduced the rate of STD by reducing the number of SVs released per AP \(\text{(Fig. 6.3, 6.8)}\). Second, using a stimulation paradigm in which two AP-like waveforms were separated by a mild depolarizing step which gradually increased \([\text{Ca}^{2+}]\), we found that, in the absence of changes in \(I_{\text{Ca}}\), SD was never prolonged suggesting that activity drives the increase in SD not simply elevations in \([\text{Ca}^{2+}]\). \(\text{(Fig. 6.13)}\). Although these data suggest that SV depletion underlies increases in SD at both the immature and mature calyx of Held, we cannot exclude the possibility that the \([\text{Ca}^{2+}]\) signal evoked during the intermediate steps may have been insufficient to significantly desensitize/inhibit the release machinery. However, since prolongations in SD can be observed following as few as one AP, in both immature and mature synapses, it is unlikely that the \([\text{Ca}^{2+}]\) signal generated during intermediate steps would be insufficient to generate a measurable decrease in SD (Fedchyshyn & Wang, 2007).

If we examine STF from the experiments shown in \(\text{Fig. 6.10 and 6.13}\), we observe that a combination of \(I_{\text{Ca}}\) facilitation and \([\text{Ca}^{2+}]-\text{dependent priming contribute to the overall potentiation of } I_{\text{EPSC}}\), however, the latter only appears effective at increasing \(P_r\) in immature synapses. Whether \(I_{\text{Ca}}\) facilitation occurs appears to depend both on the duration of ISI and whether \([\text{Ca}^{2+}]\) was elevated by applying a step, however, elevations in \([\text{Ca}^{2+}]\) may underlie both dependencies. Clearance of elevations in \([\text{Ca}^{2+}]\), resulting from a single AP, has been estimated at the immature calyx of Held to require approximately 100ms at RT and 50ms at 35°C (Helmchen et al., 1997). Since the short ISI used in our experiments is 30ms (at RT), \([\text{Ca}^{2+}]\) may be sufficiently elevated to activate \([\text{Ca}^{2+}]-\text{dependent } I_{\text{Ca}}\) facilitation, albeit to a lesser extent, even in the absence of a step. Moreover, when \(I_{\text{Ca}}\) is matched between initial and test APs, \(I_{\text{EPSC}}\) facilitation is still observed for short ISI without a step suggesting that \([\text{Ca}^{2+}]\), is elevated and capable of priming SVs in response to a single AP. In contrast, long ISI of 210ms show no \(I_{\text{Ca}}\) or \(I_{\text{EPSC}}\) facilitation in the absence of a step, in agreement with the hypothesis that elevations in \([\text{Ca}^{2+}]\), underlies both phenomena in immature synapses (Tsujimoto et al., 2002; Inchauspe et al., 2004; Ishikawa et al., 2005). This suggests that, whether from a preceding AP of short ISI or due to other elevations in \([\text{Ca}^{2+}]\), \([\text{Ca}^{2+}]\) can prime SVs and transiently increase their \(P_r\), and release kinetics, for subsequent release in immature synapses.

In contrast, mature synapses, in the absence of \(I_{\text{Ca}}\) facilitation, show no \(I_{\text{EPSC}}\) facilitation under any conditions. In the absence of a step and \(I_{\text{Ca}}\) facilitation, mature synapses show slight but significant depression in \(I_{\text{EPSC}}\) suggesting that elevations in \([\text{Ca}^{2+}]\), may serve to increase \(P_r\), slightly but insufficiently to produce \(I_{\text{EPSC}}\) facilitation or decreases in SD. These data suggest that elevations in \([\text{Ca}^{2+}]\), can produce transient increases in \(P_r\), sufficient to facilitate both \(I_{\text{EPSC}}\) and the kinetics of release, but only when loosely-coupled VGCC-SV units are present at a synapse.

Based on the above observations, we propose a model in which differences in VGCC-SV coupling provides a structural framework that predisposes synapses of different developmental stages to certain types of STP in both synaptic strength and kinetics \(\text{(Fig. 6.15)}\). We suggest that the loose and heterogeneous coupling of immature VGCC-SV units reduces \(P_r\), per VGCC opening, and allows for significant \([\text{Ca}^{2+}]-\text{dependent priming of the release machinery without SV release\). In contrast, the tight and more homogeneous coupling of mature VGCC-SV units result in a high \(P_r\), per VGCC opening,
Figure 6.15: Flow Diagram of Synaptic Vesicle Recruitment & Depletion in Immature & Mature Synapses.

(A) Flow diagram illustrating the progressive use and depletion of synaptic vesicles, in mature synapses (blue segment), leading to short-term depression of $I_{EPSC}$ and prolongation of synaptic delay. (B) Flow diagram showing the initial increase in $P_r(I_{EPSC})$ and decrease in SD, resulting from Ca$^{2+}$-dependent facilitation, and the subsequent short-term depression in $I_{EPSC}$ and prolongation in SD brought about by synaptic vesicle depletion in immature synapses (purple segment).
leaving few SVs at the AZs to be primed. This increased range in the $P_r$ of loosely-coupled SVs allows for decreases in SD to occur when $P_r$ is elevated through Ca$^{2+}$-dependent priming (Fig. 6.16 & 6.17).

Interestingly, immature and mature synapses show relatively subtle differences in their patterns of STP, in response to axon-evoked stimulation, as compared to the significant differences observed here when the AP waveform is controlled. Comparing the $I_{\text{EPSC}}$ of immature synapses in response to the AP$_I$ waveform to the $I_{\text{EPSC}}$ of mature synapses in response to the AP$_M$ waveform, at either of the $[\text{Ca}^{2+}]_o$, we observe relatively similar patterns of STP in both synaptic strength and SV release kinetics. Therefore, it appears that tightening of the VGCC-SV coupling is necessary for offsetting the coincident narrowing in AP waveform that occurs with development (Taschenberger & von Gersdorff, 2000, Yang & Wang, 2006). The wide APs in immature synapses ensure that a sufficient number of loose VGCCs open to ensure SV release, while the propensity for STF may ensure that release is maintained with minimal SD during the initial APs, which are most likely those crucial for signal processing (Abbott & Regehr, 2004). The tight VGCC-SV coupling in mature synapses ensures that $P_r$ is high upon VGCC opening, minimizing SD, despite narrow APs decreasing the $P_o$ of VGCCs. This strategy ensures that tightly-coupled SVs are available for rapid release at other AZs upon subsequent AP innervation, which ensures high-fidelity signal reproduction with minimal $I_{\text{Ca}}$. Both immature and mature synapses show pronounced STD and accompanying increases in SD later during high-frequency trains, the result of SV depletion, which may also be important for signal filtering or other processing functions (Abbott & Regehr, 2004).

Our results suggest that immature and mature synapses use fundamentally different strategies in accomplishing release of SVs which confer differing predispositions, in terms of STP and $P_r$, to each synapse. These strategies involve using a tightening in release modality to shift the limiting probability in the release cascade from the probabilistic coincidence of VGCC openings to the $P_o$ of the individual VGCCs themselves. This adaptation allows for smaller $I_{\text{Ca}}$ to effectively trigger SV release with minimal SD and increased consistency in timing and output, despite small variances in $I_{\text{Ca}}$. This is likely a key adaptation in conferring the ability of this synapses to transmit high fidelity, high-frequency signals with development.
Figure 6.16: Schematic Representation of Short-Term Plasticity in the Immature Synapse in Response to $A_P^M$ Waveforms.

(A) Illustration of five immature presynaptic AZs with associated SVs prior to the innervation of an AP.
(B-F) Illustration of SV priming and release in response to the first (B) to fifth (F) $A_P^M$ waveform.
Figure 6.17: Schematic Representation of Short-Term Plasticity in the Mature Synapse in Response to AP\textsubscript{M} Waveforms.

(A) Illustration of five mature presynaptic AZs with associated SVs prior to the innervation of an AP. (B-F) Illustration of SV priming and release in response to the first (B) to fifth (F) AP\textsubscript{M} waveform.

At Rest:
- Tightly-coupled SV-VGCC units.

AP\textsubscript{M}1:
- Many tightly-coupled SVs release on the first AP.
- Tight coupling minimizes SD

AP\textsubscript{M}2:
- High Pr leaves no SVs to be primed.

AP\textsubscript{M}3:
- Depletion of SVs lowers Pr and causes STD.

AP\textsubscript{M}4:
- STD increases.
- Recruitment of distant SVs increases SD.

AP\textsubscript{M}5:
- SVs significantly depleted.
- Extensive STD.
- Further prolonged SD.

Legend:
- SV being released
- Primed/Facilitated SV
- Unprimed SV
- VGCC
- Ca\textsuperscript{2+} Domain
- Sept5
- SNARE Proteins
7. GENERAL OVERVIEW & DISCUSSION
7.1 SUMMARY OF RESULTS

In the preceding chapters we have described a series of novel findings regarding the physical coupling between voltage-gated Ca\(^{2+}\) channels (VGCCs) and synaptic vesicles (SVs). These aimed at the characterisation of this spatial relationship during development, its effect on both the kinetics and strength of synaptic transmission, the underlying molecular determinants of VGCC-SV coupling, and its implications in generating the patterns of short-term plasticity (STP) observed in both synaptic strength and delay (SD) throughout development. We employed the calyx of Held-MNTB synapse, for its accessibility to simultaneous pre- and postsynaptic whole-cell electrophysiological recording, to address these issues.

In immature synapses (P8-10) we found that release of a SV required the cooperative interaction of many loosely-coupled VGCCs in order to bring \([\text{Ca}^{2+}]\), in the vicinity of the release machinery, to levels sufficient for engagement ("Microdomain" Modality). At this developmental stage, the VGCC population is comprised of similar numbers of N and P/Q-subtypes with similarly loose spatial distributions around SVs. Around the onset of sensory input to the system (~P11/12), each VGCC subtype experiences a different fate with N-type VGCCs leaving the active zones (AZs) and P/Q-type VGCCs coupling more tightly to the SVs. With development approaching functional maturity (~P16-18), few P/Q-type VGCCs are required to trigger release of a SV facilitated by their tight coupling ("Nanodomain" Modality).

The requirement for VGCC cooperation in triggering SV release is measured as the degree of non-linearity (\(m\) or cooperativity) in the input/output relationship of the synapse (\(\text{I}_{\text{EPSC}} \propto \text{I}_{\text{Ca}}^m\)) (Fig. 3.5). At all of the developmental stages observed, VGCCs and SVs were coupled in a distribution of tightly-coupled units (TCUs) and loosely-coupled units (LCUs), with the distribution shifting to the former with maturation. This was evident due to the incomplete attenuation (~60%) of SV release when EGTA (10mM) was loaded in immature terminals and the slight attenuation of release (~15%) still observable under the same conditions in mature synapses (Fig. 3.2). Moreover, despite a decrease in the number of VGCCs required for SV release, mature synapses showed similar maximal \(I_{\text{Ca}}\) (Fig. 3.4) suggesting that these synapses must have a greater number of AZs than in immature synapses in agreement with previous findings (Taschenberger et al., 2002). These results characterize a novel form of subsynaptic developmental adaptation which may be crucial for the development of high-frequency, high-fidelity neurotransmission (Fedchyshyn & Wang, 2005).

Repeating the experiments used to generate input-output curves for immature and mature synapses, we found that mature synapses had significantly shorter synaptic delay (SD) than did immature synapses given the same AP waveform (Fig. 5.1). Also, the dependence of SD on \(I_{\text{Ca}}\) (termed slope factor, \(s\)) was found to be markedly steeper in immature terminals than in mature ones, a trend that correlated with \(m\) for all experimental conditions in which \(m\) was manipulated (Fig. 5.2, 5.3, 5.7, 5.9). Through these experiments we were also able to demonstrate immature and mature \([\text{Ca}^{2+}]_o\)-dependencies for \(m\), in accordance with the theoretical \([\text{Ca}^{2+}]_o\)-dependence summarized by Gentile & Stanley (2004), but within the same synaptic preparation (Fig. 5.2). These data provided a correlative link between the spatial coupling of VGCCs and SVs to the kinetics of release throughout development.
Having significant functional/electrophysiological data describing the impact of VGCC-SV coupling on release, we determined through tEM that SVs were measurably further from AZs immature terminals than in mature ones on average (Fig. 5.4). This was due to an increase in the number of docked SVs at AZs. Following this we implicated Septin 5 (Sept5) as a molecular determinant of SV-AZ separation in the immature terminal by using a combination of electrophysiological recordings and tEM imaging (Fig. 5.5, 5.6, 5.7). Although a clear role for Sept5 was not ascertainable in mature synapses, we did exclude it as participating in the replenishment of the readily-releasable pool (RRP) at either developmental stage (Fig. 5.9, 5.10, 5.11, 5.12). These results provided both visual evidence and a molecular correlate for changes in the spatial coupling of VGCCs and SVs with development.

Our final series of experiments focused on investigating the effects of developmental release modality switching on short-term plasticity (STP) in synaptic strength and timing. Previously, we had characterized a novel form of temporal plasticity in mature synapses where SD and its variance progressively increased during high-frequency stimulation (Fedchyshyn & Wang, 2007). We found that the extent to which SD progressed was dependent on the stimulation frequency, [Ca\textsuperscript{2+}], and specifically elevations of [Ca\textsuperscript{2+}] within the terminal (Fig. 4.3, 4.4). Furthermore, we found that the mechanisms underlying SD progression resided downstream of Ca\textsuperscript{2+} influx and recovered with the same approximate time course as replenishment of the RRP (Fig. 4.6, 4.9). These data suggested that prolongation in SD during high-frequency transmission occurs due to the sequential depletion of SVs located close to the AZs followed by those located further and further away. By limiting [Ca\textsuperscript{2+}] build-up in the terminal and thereby limiting depletion of the RRP, the progression of SD could be minimized (Fig. 4.7).

Given these findings, we sought to determine whether differences in VGCC-SV coupling throughout development may influence the types of plasticity observed and the progression of synaptic delay. Using identical trains of high-frequency APs of varying half-widths to voltage-clamp immature and mature terminals, we found that immature synapses showed greater short-term facilitation (STF) than did mature synapses for any of the AP waveforms applied (Fig. 6.1, 6.2). During periods of STF, immature synapses also showed a transient decrease in SD that correlated with the increase in Pr generating the potentiation in SV release. As in Fedchyshyn & Wang (2007), mature terminals showed only unidirectional progression of SD for all AP waveforms (Fig. 6.6, 6.7). We were also able to correlate these STP characteristics to VGCC-SV coupling using immature low-m synapses such as those loaded presynaptically with EGTA or those taken from Sept5\textsuperscript{-/-} mice. Finally, we were able to determine that both STF and decreases in SD occur due to Ca\textsuperscript{2+}-dependent “priming” of the release machinery transiently increasing Pr, a process which is enabled by loose VGCC-SV coupling. In contrast, STD and increases in SD were found to be caused by depletion of the RRP, a process that is more rapid when VGCCs and SVs are tightly-coupled for a given AP waveform (Fig. 6.14).

The above data led us to propose a “positional” model in which the location of a SV relative to its VGCCs primarily determines its Pr at the calyx of Held-MNTB synapse. In this model, loose VGCC-SV coupling results in a population of SVs with heterogeneous Pr, which depend on their spatial intimacy with VGCCs, the stochastics of their opening, and cooperation in generating I\textsubscript{Ca} upon AP invasion. This heterogeneity allows for bidirectional modulation of Pr, such that STF, and decreases in SD, are possible.
given an appropriate non-saturating input signal. In contrast, mature synapses use a more homogeneous array of VGCC-SV spacing in which SV release depends heavily on the activation of few closely coupled VGCCs, which results in a much higher $P_r$ for a given AP waveform. This release modality exhibits monotonic decreases in $P_r$ as SVs are rapidly depleted and recruitment of more distant SVs occurs.

Based on these results, we propose that a conversion from a micro- to nanodomain release modality produces a more consolidated mature synapse with more stable firing patterns, shorter SD, and reduced dynamic range. These subsynaptic adaptations could be crucial for the development of high-fidelity synaptic transmission, such as that characteristic of the mature calyx of Held-MNTB synapse. In addition, it may in part determine the patterns of short-term plasticity expressed.

7.2: THE CALYX OF HELD-MNTB SYNAPSE AS A MODEL SYSTEM

The calyx of Held-MNTB synapse is an ideal system in which to investigate presynaptic neurophysiology in the mammalian brain. Its usefulness, as it pertains to the preceding chapters, is owing to three primary characteristics; experimental ability, extendibility of findings (its commonalities with other synapses), and for the extensive background knowledgebase that has been generated since the establishment of the preparation for presynaptic electrophysiological recordings (Forsythe, 1994; Takahashi et al., 1996; Helmchen et al., 1997; Borst & Sakmann, 1998).

7.2.1 Technical Advantages & Abilities.

Due to its large size, continuous surface, and electrically compact structure, the calyx of Held-MNTB synapse is one of the few mammalian central synapses in which high-quality, whole-cell electrophysiological recordings can be made. These features allow for voltage- and current-clamp experiments (Forsythe, 1994; Takahashi et al., 1996), capacitance measurements (Sun et al., 2004), Ca$^{2+}$ imaging and uncaging (Helmchen et al., 1997; Schneggenburger et al., 1999), and loading of foreign compounds to be performed individually or in simultaneous combination with similar techniques in the postsynaptic MNTB neuron (Takahashi et al., 1998; Yamashita et al., 2003; Fedchyshyn & Wang, 2005). However, the experimental accessibility of the terminal comes at the price of rapid dialysis of endogenous mobile constituents upon whole-cell perturbation. We found that dialysis of presynaptic intracellular solution (ICS), without the supplementation of glutamate, resulted in rapid (~1min.) rundown of $I_{EPSC}$ (data not shown). This suggests that the environment of the terminal becomes relatively homogeneous following innervation and questions whether the presynaptic environment remains “physiological” in the context of certain experiments. Curiously, previous studies have omitted glutamate completely or added far in excess of 3mM (see section 2.2.2) to the ICS but documented no rundown or potentiation of $I_{EPSC}$ despite reasonable quoted input resistance criterion (i.e. Borst & Sakmann, 1996). Interpretation of results investigating the effects of second messengers or other endogenous mobile elements should be cognisant of this rapid dialysis.
Recent work employing the calyx of Held has also shown its value for use in imaging experiments using either confocal microscopy or transmission electron microscopy (TEM) from slice preparations (Rowland et al., 2000; Sätzler et al., 2003; Leão et al., 2005; Wimmer et al., 2006; Nakamura & Takahashi, 2007). These high-quality images have allowed for high-resolution 3D reconstructions of the calyx of Held and have provided insight into the morphological features unique to this synapse and into those of synapses in general (see below).

While the above techniques have proven fruitful in their generation of novel data, other techniques such as quantitative immunohistochemistry, typical protein assays, or imaging via cell culture has been much less so. The low density of calyx of Held-MNTB synapses, as compared to the high density of synapses within the hippocampus for example, results in low signal to noise ratio (SNR) in biochemical assays from prepared tissue. This is particularly true with maturation as the number of synapses, or at least MNTB neurons, appears to decrease significantly after the onset of hearing (~P11). Moreover, to date, no successful culture system has been able to express viable calyces and MNTB neurons, which has limited the widespread use of viral transfection techniques and higher resolution imaging (also see Wimmer et al., 2004). A culture system of this type would allow for facilitated experimentation, use of more cost-effective experimental equipment, and allow for a host of lower-level experimental targets with longer experimental durations. Simply achieving sustainability of such a culture system may also be instructive for understanding the minimal environmental ingredients required for the development of these specialized neurons.

The calyx of Held in the rodent brainstem has also been extremely beneficial in the functional elucidation of genetic modifications on synaptic transmission. P/Q and L-type VGCCs (Inchauspe et al., 2004; Erazo-Fischer et al., 2007), synapsin 1&2 (Sun et al., 2006), NCS-1 (Tsujimoto et al., 2002), Sept5 (Chapter 5), CSPα (Fernández-Chacón et al., 2004), and parvalbumin (Müller et al., 2007) KO synapses have all been used to investigate the roles of these proteins and/or synaptic transmission with higher experimental resolution than at any other central synapse.

There are two primary disadvantages to using the calyx of Held-MNTB in concert with genetically altered animals. The narrow developmental/experimental window makes investigation of embryonic-lethal mutations impossible to study in a functional context. This is due to the late contact and development of the calyceal nerve terminal onto the MNTB neuron occurring no earlier than P2 (Hoffpaurir et al., 2006). Also, there are no currently identified cell-specific promoters that can target gene disruption to only the calyx, through the globular bushy cells (GBCs), or to the MNTB neurons specifically. Because of these difficulties in synapse-specific genetic disruption, the mechanisms underlying the impact of genetic manipulation on higher-level functions should be carefully extrapolated from data collected from the calyx of Held-MNTB synapse. Likewise, secondary effects on development of the calyx, due to potential dysfunction in other regions, must be considered in the context of such experiments.

Despite limitations, which are present in any experimental system, the calyx of Held-MNTB synapses offers an incredibly powerful tool for the investigation of presynaptic neurophysiology and will certainly provide further insights into this difficult topic of study. Indeed, few of the results present herein
could have been generated from any other synapse in the CNS. Recently, whole-cell recordings from mossy-fibre terminals in the CA3 region of the hippocampus have been made providing an alternative location at which to directly study presynaptic neurotransmission. However, the small currents and low experimental efficiency limit the widespread use of this preparation (Geiger & Jonas, 2000).

### 7.2.2 Extendibility & Transferable Knowledge.

As with any specialized/unique synapse, the degree to which knowledge gained from it is transferable determines its usefulness for generalization to systems more relevant for pathological conditions or phenotype expression. The calyx of Held is extremely unique morphologically as compared to more conventional arborized synapses in the CNS (Held, 1893; Ramón y Cajal, 1972; Morest, 1968). While conventional synapses extend their presynaptic AZs into spatially distinct locations so that they may be activated and modulated individually, the calyx of Held has placed all of the AZs in a single, relatively uniform, compartment. These arrangements differ functionally in that AZs at conventional synapses are generally activated by a number of APs, from different sources, which are integrated into a postsynaptic response. Only a single AP innervates the calyx through the giant axon of the GBC, uniformly activating all AZs and performing all of the synaptic integration on the input/presynaptic side of the synapse. However, the calyx of Held-MNTB synapse is comprised of a relatively typical set of synaptic ingredients suggesting that at least neurotransmitter release should operate in a typical manner. Typical ion channels, release machinery proteins, structural elements, and signalling complexes all exist at this synapse and appear to function in similar ways to those at other CNS synapses (Schneggenburger & Forsythe, 2006).

Ironically, most other CNS synapses do not allow for the resolution of study afforded by the calyx of Held making determination of what constitutes “normal” functionality at the calyx of Held-MNTB synapse paradoxical. Subsynaptic structure (i.e. AZs) appear to be relatively similar between synapses providing some, albeit static, evidence that this synapse is structured like its more conventional neighbours (Sättler et al., Hoffpaur et al., 2006). However, the calyx of Held also has some atypical features. For example, Na\textsubscript{V}1.2 Na\textsuperscript{+} and Kv1.2 K\textsuperscript{+} channels in the calyx are located only in the region where the axon meets the calyceal terminal, called the heminode, which is thought to ensure rapid and consistent spread and repolarization of the incoming AP throughout the terminal (Dodson et al., 2002; Leão et al., 2005). Despite the examples above, the largest barrier to the widespread adoption of the calyx of Held as the CNS model system is its traditional lack of expression of typical forms of long-term plasticity (LTP), such as LTP and LTD, like those observed throughout the hippocampus (Bliss & Lømo, 1973). While some novel forms of long-lasting activity-dependent potentiation of \text{I\textsubscript{EPSC}} have been observed (Habets & Borst, 2005; Lou et al., 2005; Habets & Borst, 2006; Habets & Borst, 2007), they do not appear to share common mechanisms with classical LTP. Moreover, due to its single neuron innervation and distant contacts with other synapses (i.e. LSO, GBC), our understanding synaptic integration and network phenomenon are less likely to benefit directly from experiments conducted at the calyx than in other CNS preparations. More recent studies, performed in immature calyx of Held-MNTB synapses, suggest that under appropriate pre- and post synaptic conditions, a form of LTD can be
triggered, signifying that perhaps this synapse requires different stimulation in order to exhibit long-term plasticity (Joshi et al., 2007). Further investigation is ongoing to determine the mechanisms underlying expression of this novel plasticity (Yang et al., unpublished work). Despite the young nature of these studies at this synapse, our current understanding of its functionality may provide insights into new forms of short-term plasticity at single-neurons which may be crucial for the filtering/modulation of information at individual synaptic relays (Thomson, 2000; Abbott & Regehr, 2004).

Much as the establishment of the squid giant synapse and rodent or frog neuromuscular junction preparations revolutionized understanding of synaptic transmission, the calyx of Held has afforded us the extension and resolution of those seminal studies into the mammalian brain.

7.2.3 Extensive Existing Knowledgebase & Novel Paths of Study.

The calyx of Held-MNTB synapse has been extensively studied in a relatively short period due to its accessibility, short well-defined period of development, and simple architecture (for reviews see von Gersdorff & Borst, 2002; Schneggenberger et al., 2002; Schneggenberger & Neher, 2005; Schneggenberger & Forsythe, 2006). These features provide a series of experimental paths in which the Calyx of Held-MNTB synapse is a useful model system.

The observation that the calyx of Held develops its ability to transmit high-frequency signals with high-fidelity over such a short period during development, in concert with the onset of activity in the system (~P11) (von Gersdorff & Borst, 2002), raises an interesting question. Do the synaptic modifications that result in this ability arise due to the onset of activity (i.e. activity-dependent) or are they programmed through genetics to occur regardless of input (i.e. activity-independent)? Further complicating this question is the fact that genetic programming can be altered by environmental factors (i.e. epigenome) such as activity, and that activity may act in concert with genetics to manifest as any one of the many characteristic synaptic changes observed at the calyx of Held. By eliminating the input to the calyx of Held, using genetically deaf mice, cochlear ablation, or other techniques, the roles of both genetics and activity can be investigated and separated in the context of synaptic development and high-frequency capability (Erazo-Fischer et al., 2007; Oleskevich et al., 2004). Using any of these techniques we could trace the development of the nanodomain release modality, using experiments such as those in Fedchyshyn & Wang 2005, and determine whether it occurs in the absence of activity. These experiments could suggest whether tightening of the release modality requires a synaptic signal for initiation, and if so, the key players in signalling this change could be dissected through pharmacological or genetic means. Alternatively, tightening of the release modality could be completely independent of activity, in which case experimentation focused on the elucidation of its expression should be targeted at changes in gene/protein expression. Luckily, the calyx itself is most accessible to experimentation during the short window of development during which release modality tightens, allowing for efficient collection of data and yielding associated results relatively rapidly.

Besides investigation focused on development, the calyx of Held-MNTB synapse provides a system in which excitation/secretion coupling can be studied through detailed modelling. This is facilitated by the large number of known synaptic parameters owing to the popularity and accessibility of
the preparation. Accurate models can be incredibly useful for anticipating the subtleties of synaptic transmission that may be inaccessible to direct experimental measure. For example, in the absence of freeze-fracture EM imaging, which has been successfully performed at the frog NMJ, the exact topology of VGCCs and SVs remains unknown at the calyx of Held terminal (Hirokawa et al., 1989; Harlow et al., 2001). Elegant modelling studies by Meinrenken et al., (2002, 2003) have hypothesized the arrangement of VGCCs and SVs based on the current inventory of known synaptic parameters and experimental data. Since this work, new information has surfaced clarifying some of the previously unknown model parameters such as endogenous slow buffer concentration and kinetics (Müller et al., 2007) which make future refinements possible. Unfortunately, to date, experimental models have used parameters and experimental data acquired exclusively from immature synapses, most likely due to the relative experimental ease in which this data can be generated. This has resulted in extrapolation of the particulars of these models beyond the scope of the experiments upon which they were generated. As discussed here and elsewhere, maturation beyond the typical experimental ceiling of P12 is accompanied by a series of robust synaptic changes that may render models based on immature synapses inaccurate or misleading (von Gersdorff & Borst, 2002). Similar rigour should be applied to building accurate models of mature synapses as has been done in the immature case (see section 9.3).

These models, and the experimental evidence generating their parameter values, will further our understanding of the fundamental changes that occur in excitation/secretion coupling with development and what synaptic advantages they may confer. They may also allow insight into the “design” of the presynaptic CNS AZ and release face until direct visualization of these elements can be achieved.

SECTION 7.3: IMPLICATIONS OF RELEASE MODALITY TIGHTENING ON SYNAPTIC SIGNAL TRANSDUCTION

7.3.1 Cooperativity & Sources of Non-Linearity in Excitation/Secretion Coupling.

The term “cooperativity” is used to describe non-linearities in the input-output relationship of a synapse. These may arise from a variety of processes within the neurotransmission cascade. Interestingly, under physiological conditions, presynaptic non-linearities generally amplify variations in input signals and expand the dynamic range of release, while postsynaptic non-linearities have an attenuating effect on the reception of that release. For example, postsynaptic AMPARs can be saturated and/or desensitized during repetitive activity, attenuating the true range of presynaptic output (Otis et al., 1996a; Otis et al., 1996b; Scheuss et al., 2002; Taschenberger et al., 2002; Taschenberger et al., 2005). While the purpose of these reception limiting mechanisms are not completely understood, it is well documented that they do not persist with significant strength into synaptic maturation and have little impact on the transmission of single or bursts of APs (Taschenberger et al., 2002; Yamashita et al., 2003). These findings imply that the development of high-fidelity neurotransmission requires that factors downstream of release be linearized for maximal synaptic efficacy. Here we have focused on the
aspects of presynaptic excitation/secretion coupling that underlie the high-power relationships observed between Ca\(^{2+}\) and SV release.

“Molecular cooperativity” refers to the cooperative binding of multiple Ca\(^{2+}\) ions, with estimates ranging from 3 to 5, to the Ca\(^{2+}\) sensor, generally accepted as synaptotagmin (Dodge & Rahamimoff, 1967). The resultant power relationship describes the dependence of release on the driving force for Ca\(^{2+}\) into the terminal through a given subset of available VGCCs. While these studies have allowed us to better understand the properties of the Ca\(^{2+}\) sensor, there has been very little variation in measurements of this quantity across a variety of systems, suggesting that the sensors properties are relatively conserved (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986; Stanley, 1986; Landó & Zucker, 1994; Heidelberger et al., 1994; Reid et al., 1998; Bollmann et al., 2000; Qian & Nobels, 2001). However, molecular cooperativity may not have such a straight forward physical interpretation. While it is well documented that the binding of Ca\(^{2+}\) ions to synaptotagmin is cooperative, it is not clear whether engagement of multiple SNARE complexes, around the base of a SV, is also cooperative which would increase molecular cooperativity (Stewart et al., 2000). In fact, any Ca\(^{2+}\)-dependent step necessary for the release of SVs is expected to contribute to the measured molecular cooperativity. Thus, retaining the sole view that molecular cooperativity refers to the binding of Ca\(^{2+}\) ions to synaptotagmin may be largely historical (Dodge & Rahamimoff, 1967).

Under conditions of constant Ca\(^{2+}\) driving force (i.e. constant [Ca\(^{2+}\)]\(_o\)), molecular cooperativity can be separated from that investigated here termed “Ca\(^{2+}\)-domain cooperativity” or \(m\) (Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005; Shahrezaei et al., 2006). Ca\(^{2+}\)-domain cooperativity refers to the power relationship between the number of VGCCs/Ca\(^{2+}\) domains producing a Ca\(^{2+}\) signal and SV release. Interestingly, unlike molecular cooperativity, Ca\(^{2+}\)-domain cooperativity varies highly in different model systems and appears to be due to a combination of the spatial separation of VGCCs and SVs and the native [Ca\(^{2+}\)]\(_o\) of the system (Llinas et al., 1981; Brandt et al., 2005; Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005; Shahrezaei et al., 2006). Systems in which [Ca\(^{2+}\)]\(_o\) is high and/or VGCC-SV coupling is tight are found to have \(m\)-values of approximately 1. These include the squid giant synapse with physiological [Ca\(^{2+}\)]\(_o\) of ~10mM and the chick calyceal synapse with [Ca\(^{2+}\)]\(_o\) of ~5mM (Llinas et al., 1981; Gentile & Stanley, 2005). In contrast, the murine calyx of Held synapse has an [Ca\(^{2+}\)]\(_o\) of ~1.5mM and can exhibit \(m\)-values between 2 and 6 depending on the developmental stage (Fedchyshyn & Wang, 2005). Increasing [Ca\(^{2+}\)]\(_o\) at this synapse decreases \(m\)-values due to larger individual Ca\(^{2+}\) domains reducing the requirement for extensive Ca\(^{2+}\) domain overlap in generating suprathreshold Ca\(^{2+}\) signals. Conversely, decreasing [Ca\(^{2+}\)]\(_o\) has the opposite effect, decreasing the size of individual Ca\(^{2+}\) domains, and increasing the need for Ca\(^{2+}\) domain overlap (Fig. 5.2D). The range of \(m\)-values that can be measured from a single synapse is instructive as it describes the properties of the Ca\(^{2+}\) sensor at very small [Ca\(^{2+}\)]\(_o\) and describes the minimal synaptic unit at high [Ca\(^{2+}\)]\(_o\) (Stanley, 1993; Gentile & Stanley, 2005).

Experimentally, it is impossible to acquire reliable data at very high/low values of [Ca\(^{2+}\)]\(_o\), although extrapolation of “reliable” data can be theoretically provoking. At very low [Ca\(^{2+}\)]\(_o\), Ca\(^{2+}\) driving force is insufficient to generate suprathreshold Ca\(^{2+}\) signals preventing SV release and making it impossible to
measure $m$. At very high $[Ca^{2+}]_o$, rapid saturation of individual VGCCs upon AP waveform broadening becomes a limiting factor in generating input-output curves with a variable enough range of input values for fitting.

At the upper limit of $[Ca^{2+}]_o$ found in nature, $m$-values approach 1, implying that a single VGCC is capable of triggering release of a single SV and forming a minimal functional unit (Stanley, 1993; Bertram et al., 1996; Stanley, 1997; Gentile & Stanley, 2005). A minimal $m$-value of 1 also implies that all SVs, and their associated VGCC, operate independently of other VGCC-SV units and that each AZ is independent of other AZs which is a necessary condition in the evaluation of the number of release sites by certain methods (Elmqvist & Quastel, 1965; Quastel 1997; Meyer et al., 2001). However, $m$-values of less than 1 are occasionally measured in these systems implying that the Ca$^{2+}$ signal from one VGCC may be capable of triggering release of more than one SV (Gentile & Stanley, 2005). This finding argues that AZs, or at least release sites, are not independent and calculations of their number must consider this possibility in some systems. In addition, a lower limit for $m$ of less than 1 raises additional important issues surrounding interactions between VGCCs and release proteins (i.e. synprint region and association binding partners), release site topology, and the observed species/system differences.

Under these conditions, SV release would actually have a resistance to changes in Ca$^{2+}$ input which could be advantageous in processing tasks where dynamic range must be minimized (i.e. relays) (Abbott & Regehr, 2004). However, the rarity of $m$-values this low suggests that they are an exception amongst minimal synaptic units that generally contain multiple VGCCs per SV.

At the lower limits of $[Ca^{2+}]_o$, it is thought that the maximal $m$-value should refer to the number of Ca$^{2+}$ binding sites on the Ca$^{2+}$ sensor (Gentile & Stanley, 2005). That is, $\alpha$ binding sites can at most be filled by Ca$^{2+}$ ions from $\alpha$ discrete VGCCs, placing an upper boundary condition on $m$. Interestingly, modeling at the calyx of Held has required as many as five Ca$^{2+}$ binding sites to explain experimental observations while $m$-values have been recorded as high as ~6 (Bollmann et al., 2000; Fedchyshyn & Wang, 2005). Based on our structural understanding of the C$_2$A and C$_2$B Ca$^{2+}$ binding domains of synaptotagmin, five Ca$^{2+}$ ions is the highest number that can possibly bind to an individual synaptotagmin molecule (Shao et al., 1998; Fernandez et al., 2001). So why then do we observe $m$-values higher than 5 when Ca$^{2+}$ is lowered or when VGCC-SV coupling is spatially distant (i.e. immature synapses)? Furthermore, how can estimates of the number of VGCCs required to release a single SV range from 12-60 in the same synapse (Borst & Sakmann, 1996; Schneggenburger & Neher, 2000; Meinrenken et al., 2002)? The most likely explanation is that more than one synaptotagmin/SNARE complex is required for SV fusion, each requiring 4-5 bound Ca$^{2+}$ ions (see Stevens & Sullivan, 2003).

For stability of a spherical structure on a flat surface, a minimum of three support points are required. If we consider this to be the minimal arrangement for the stable docking of an SV at the presynaptic release face, then fusion should require the binding of 12-15 Ca$^{2+}$ ions to engage the array of Ca$^{2+}$ sensors. It is then possible that up to 15 distinct VGCCs could contribute to vesicular release considering the “minimal” structural arrangement. However, this does not exclude the possibility that activation of more than three synaptotagmin/SNARE complexes may underlie the membrane fusion step, increasing the upper limit for $m$ further. The estimated number of SNARE protein complexes required for
fusion of SVs has ranged between 3 and 15 depending on the estimate of the energy barrier to fusion (Weber et al., 1998; Jahn et al., 2003; Montecucco et al., 2005; Jahn & Scheller, 2006). Consideration of this number of synaptotagmin molecules greatly expands the potential upper limit for \( \text{Ca}^{2+} \)-domain cooperativity.

The number of SNARE complexes involved in holding a docked SV can also determine the distance at which the SV resides in reference to the AZ membrane. At very small distances (∼10nm), SV membranes experience repulsion due to surface charge resident on the AZ membrane. Complexing of v-SNARES and t-SNARES overcomes this repulsion and brings SVs to within nm of the AZ membrane. However, the number of SNARE complexes which interact determines the distance at which a SV will sit while docked at a particular AZ (Yersin et al., 2003). For example, if one or two SNARE complexes form, a SV will dock but remain at a distance of ∼15-20nm from the AZ membrane (Yersin et al., 2003). If more SNARE complexes form, this distance will decrease thereby decreasing the distance of the SV to the VGCCs. In this sense, the number of SNARE complexes may not only define the ceiling for molecular cooperativity, but it may also determine, in some part, the separation between VGCCs and SVs thereby defining \( \text{Ca}^{2+} \)-domain cooperativity.

Our experiments involving immature Sept5−/− synapses show a decreased \( m \)-values and SD (Chapter 5). It is possible, given the relationship between the number of SNARE complexes and the SV-AZ separation distance, that Sept5 may interfere with a subset of the SNARE complexes located at any given SV. In this arrangement, binding of Sept5 to syntaxin would eliminate those SNAREs from complexing and increase the “stretch” of those remaining. Thus, fusion time would increase upon \( \text{Ca}^{2+} \) influx, and perhaps more pull by synaptotagmin molecules would be required to fuse the SV. While there is no current proof for Sept5 working in this way, it is a possible scenario for explaining our experimental findings.

The fact that most experimental evaluations of \( m \) do not surpass 5 may indicate that these measurements generally reflect a unit average number of VGCCs per \( \text{Ca}^{2+} \) sensor rather than per SV. In addition, the spatial restriction imposed by a SV on the spread of \( \text{Ca}^{2+} \) ions may restrict \( \text{Ca}^{2+} \) contributions from VGCCs to the geometric side of the SV in which they reside (Shahrezaei & Delaney, 2004). Finally, most modelling, including that in appendix 9.3, of the \( \text{Ca}^{2+} \) sensor require 5 binding sites in order to accurately predict experimental observations. This suggests that the properties of synaptotagmin likely underlie this consistent finding.

By pushing experimental evaluation of \( m \) into domains of higher and lower [\( \text{Ca}^{2+} \)], than those investigated here, we could determine, in the same synapse, where the limits for \( m \) reside. These data would allow for appropriate boundary conditions to be applied to models describing excitation/secretion coupling at this synapse and the further interpretation of the \( m \)-values observed in other systems. In addition, future biochemical experimentation should attempt to determine the number of synaptotagmin molecules that are resident on each SV, which would help to understand the exact quantity being measured as “molecular cooperativity”.

The fact that synapses with a variety of \( m \)-values, are effective at performing their associated functions suggests that shifts in the interplay between VGCC-SV localization and the driving force for
Ca$^{2+}$ may confer different properties/abilities under different conditions. Thus, choosing differential spatial coupling of VGCCs and SVs, in concert with different [Ca$^{2+}$], may provide subsynaptic control of release probability and the kinetics of release.

### 7.3.2 Release Probability & Synaptic Delay – Micro- vs. Nanodomain Release Modalities.

Release of SVs is ultimately a probabilistic event that relies on a few primary steps, also probabilistic, to culminate in vesicular fusion. First, upon invasion of an AP into the nerve terminal, VGCCs open depending on the amplitude and duration of the AP waveform, producing local domains of elevated Ca$^{2+}$. The shape and summation of the Ca$^{2+}$ domains will depend on the pattern of chance openings of individual VGCCs within the AZ. This Ca$^{2+}$ signal diffuses through the cytoplasm where its shape and spread are defined by endogenous buffers and fixed structures (i.e. SVs, proteins) (Zhou & Neher, 1993; Klingauf & Neher, 1997; Naraghi & Neher, 1997; Ohana & Sakmann, 1998; Meinrenken et al., 2003; Shahrezaei & Delaney, 2004). Next, the Ca$^{2+}$ domain will spread to reach the Ca$^{2+}$ sensor where it is either supra- or subthreshold in satisfying the binding affinity of the sensor. Whether a SV is released will depend on its presence at the AZ, the Ca$^{2+}$ affinity of the sensor, and the completeness of the release apparatus (for reviews see Lin & Sheller, 2000; Südhof, 2004; Becherer & Rettig, 2006). Ultimately, whether a Ca$^{2+}$ signal is sub or suprathreshold depends on how the signal was generated and what interactions it has undergone in transit to the sensor. Obviously, the distance of VGCCs from the SV will significantly impact the nature of the Ca$^{2+}$ signal and therefore the overall probability of release ($P_r$).

At the calyx of Held, we can observe how two functionally adequate but physically distinct arrangements of VGCCs and SVs modulate the probabilistic steps leading to SV release. Furthermore, by controlling the AP waveform these steps can be somewhat parsed. In order to ensure suprathreshold Ca$^{2+}$ signals, immature synapses utilize wide APs to bring more VGCCs into the open state. These contribute to large Ca$^{2+}$ signals which can traverse longer distances, at higher concentrations, and reach distant SVs triggering release. Thus, the key factor in the success of the microdomain modality is that a large number of VGCCs are probabilistically activated, in the vicinity of a SV, to generate large Ca$^{2+}$ transients.

In contrast, mature synapses using the nanodomain modality, employ narrow APs which reduce the open probability of individual VGCCs significantly lowering the release probability. This is compensated for by the tight localization of these VGCCs, making smaller Ca$^{2+}$ signals more effective in reaching threshold at the sensor. In this modality, the primary determinant of SV release is whether the narrow AP is of sufficient duration to activate a tightly-coupled VGCC. Given the above, evaluations of changes in $P_r$, should consider under what specific conditions these changes actually occur. For example, it has been well documented that $P_r$ decreases at the calyx of Held with development (Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001), however for a given input, the $P_r$ increases dramatically with development (Fig. 3.1) (Fedchyshyn & Wang, 2005).

While it is clear that the micro- and nanodomain modalities utilize very different strategies to manage their global $P_r$, the advantages that each has on excitation/secretion coupling is instrumental for
understanding the development of high-fidelity neurotransmission. The nanodomain release modality confers two primary advantages over the microdomain modality during single AP events. First, it requires less Ca²⁺ overall to generate an equivalent output signal than the microdomain modality. This is advantageous as, due to endogenous buffering, it readily compartmentalizes Ca²⁺ signals to a narrow volume within the AZ and effectively separates neural signalling from other Ca²⁺-signalled cellular processes (Naraghi & Neher, 1997). Secondly, and examined extensively herein, the nanodomain modality reduces the delay required to occupy the Ca²⁺ sensor and initiate the fusion process (SD) (Chapter 5).

Recent evidence has shown that the Ca²⁺ signal required to evoke release from the immature calyx terminal is very brief (~0.5ms) and of substantially lower elevation than previously thought (~10-20μM vs. 200μM) (Bollmann et al., 2000; Schneggenburger & Neher, 2000; Yamada & Zucker, 1992). This implies that the Ca²⁺ sensor is more sensitive than originally thought, capable of monitoring even small increases in [Ca²⁺]. In addition, binding of Ca²⁺ to the sensor appears to be the limiting factor in determining the kinetics of release (Felmy et al., 2003). In agreement with our data, showing that SD depends on the degree of VGCC cooperation in generating a Ca²⁺ signal, this suggests that the rise and fall of the local Ca²⁺ signal, and the upstream factors underlying it, determine the kinetics of SV release in addition to its magnitude (Fig. 5.8). Thus, establishing a sharp [Ca²⁺] gradient close to the release apparatus would be an effective means of decreasing SD and taking advantage of the high-sensitivity of the release machinery. Indeed, when AP waveform is held constant, mature synapses have a much shorter SD than do immature synapses (Fig. 5.1). The longer delay observed in immature synapses is owing to a combination of increased transit time for Ca²⁺ ions to reach the Ca²⁺-sensor and the requirement for temporal summation of many VGCC openings in triggering release (Chapter 5). In addition, it is likely that, in the absence of disruption to the presynaptic terminal during whole-cell recording, increased Ca²⁺ buffering would further constrain and delay the spread of the Ca²⁺ signal over the increased distances present in immature synapses. This reduction in [Ca²⁺] may also increase the SD in these synapses. However, under the experimental conditions presented herein, mobile buffer concentrations are likely controlled across developmental groups through dialysis of exogenous buffers with those contained in the presynaptic recording pipette, into all terminals. Given this, the observed differences in SD across experimental groups most likely arise from consequences of different release modalities (Fedchyshyn & Wang, 2005).

In an attempt to provide proof of principle regarding how VGCC-SV coupling can explain the observed difference in SD between immature and mature synapses, we have reconstructed existing models of buffered Ca²⁺ diffusion and the release sensor from the calyx of Held (Naraghi & Neher, 1997; Bollmann et al., 2000). Combining these models, we have simulated the impact of developmental release modality tightening on both the kinetics and strength of SV release (Appendix 9.3). The results of this experiment suggest that approximately 75% of the observed delay can be explained solely due to changes in VGCC-SV coupling and the resulting changes to Ca²⁺ dynamics within the terminal. These data provide further confirmation that changes in VGCC-SV coupling are sufficient to explain our observations regarding SD changes during development.
Interestingly, when we compare the dependence of $I_{\text{EPSC}}$ and $SD$ on $I_{\text{Ca}}$ ($m$ and $s$ respectively), we find that while they are strongly correlated, they do not share the same power relationship. $SD$ is linear in $I_{\text{Ca}}$ while $I_{\text{EPSC}}$ is a power relationship in $I_{\text{Ca}}$ (Chapter 5 & 3 respectively). So how then can cooperativity and slope factor be correlated through release modality and $P_r$? The first step in rationalizing this apparent discrepancy may be to analyze the nature of the experimental paradigms generating the data. In the case of cooperativity, we assay the global release as a function of the number of VGCCs contributing to the $Ca^{2+}$ domains. The total release is a measure of those SVs at which the contribution of the VGCCs was sufficient to generate a suprathreshold $Ca^{2+}$ signal. It is a time-integrated quantity that contains no information besides the binary supra/subthreshold condition for the composite SVs. In this sense, this assay does not measure the subtleties of release but rather whether and to what extent it occurred for a given number of VGCCs recruited. Thus, whether $[Ca^{2+}]$ in the vicinity of a SV is suprathreshold, is a power function of the number of VGCCs recruited with a variable exponent ($m$) depending on their localization (Fedchyshyn & Wang, 2005; Gentile & Stanley, 2005).

In contrast, analysis of slope factor ($s$) determines the delay in release of the fastest SVs of the population that experienced suprathreshold $[Ca^{2+}]$. If the rate limiting step for fusion is the binding of $Ca^{2+}$ ions to synaptotagmin, then the dependence of $Ca^{2+}$ binding rates on the $[Ca^{2+}]$, should also dictate the dependence of $SD$ on $[Ca^{2+}]$. Since $[Ca^{2+}]$, is determined by the pattern and proximity of VGCC openings in relation to a SV, release modality would influence the binding kinetics of $Ca^{2+}$ ions to the $Ca^{2+}$ sensor (Felmy et al., 2003; Schneggenburger & Neher, 2000; Bollmann et al., 2000; Bollmann & Sakmann, 2005).

It would appear then that the binding kinetics of $Ca^{2+}$ ions to the $Ca^{2+}$ sensor is linearly dependent on the number of VGCCs recruited while the recruitment itself is non-linearly dependent on the same. However, others have observed a non-linear relationship between $SD$ and $[Ca^{2+}]$, generated through flash photolysis of caged-$Ca^{2+}$ compounds in immature synapses, in contrast to our observations here (Bollmann & Sakmann, 2005). One explanation for this apparent discrepancy is the large range of $[Ca^{2+}]$, generated in these experimental paradigms which evoked a range of $SD$ 5 to 10-fold larger than those evoked and observed herein. While under our experimental conditions we cannot know the exact $[Ca^{2+}]$, we can infer the difference based on the observed changes in $SD$ in relation to those observed by Bollmann & Sakmann (2005). Given these technical differences, it is possible that the relationship between $I_{\text{Ca}}$ and $SD$ is in fact non-linear, but only over a range of $[Ca^{2+}]$, well above that evoked here. Thus, we may be sampling a relatively small portion of the $SD-I_{\text{Ca}}$ curve which has an apparent curvature within error of linearity over the present experimental range. This also suggests that, within a range of $I_{\text{Ca}}$ expected based on physiological forms of modulation (i.e. GPCRs), $SD$ most likely changes in a relatively linear manner.

The different slope factors ($s$), observed to correlate with $m$, may arise from the $Ca^{2+}$-sensor experiencing different levels of suprathreshold $[Ca^{2+}]$ based on the different localization of the VGCCs to the SV. With the relationship between $I_{\text{Ca}}$ and $SD$, across a large range of $[Ca^{2+}]$, being non-linear, we would expect a change in the apparent slope of this relationship correlating with the $[Ca^{2+}]$, and the point...
on the $[Ca^{2+}]$-SD curve to which it corresponds. Since $I_{EPSC}$, and thus $m$, also depends on $[Ca^{2+}]$, this may explain how both $m$ and $s$ appear linked through $P_r$.

In low-$m$ synapses, tightly-coupled VGCCs may establish $Ca^{2+}$ domains that are well above threshold for release and near the shortest possible values for SD. The additional contribution of more VGCCs, upon AP waveform broadening, may increase $[Ca^{2+}]$, further, but may have minimal impact on SD, minimizing the headroom for resultant decreases in SD. When averaged across all AZs for a given range of VGCC recruitment (i.e. $AP_0$ paradigm Fig. 3.5), the smaller number of VGCCs necessary for triggering SV release produce a smaller potential range for $[Ca^{2+}]$, which, while suprathreshold, would result in a smaller range of SD (i.e. shallow slope factor). Given the increased number of AZs in mature synapses, and the relatively constant maximal $I_{Ca}$ observed throughout development (Fig. 3.4), this could explain the difference in $s$-values measured and their correlation with $m$. Thus, the range of SD measured may reflect the ranges of $[Ca^{2+}]$ experienced by SVs. Moreover, the use of high $[Ca^{2+}]$ to increase release kinetics may partially explain the high safety-factor observed at this synapse. When these data are considered together, the establishment of the $Ca^{2+}$ domain, and its dynamics within the AZ, are the key events through which the kinetics and strength of release are determined.

As discussed above, the location of the $Ca^{2+}$ source will dictate not only the magnitude of $I_{Ca}$ required to trigger release but also whether factors affecting $Ca^{2+}$ ion transit (i.e. fast and slow buffers) will have an effect on release at all (Naraghi & Neher, 1997; Meinrenken et al., 2003). Interestingly, many of the presynaptic modulatory pathways, including activation of GPCRs or mGluRs, selectively target $I_{Ca}$, and thus the nature of the $Ca^{2+}$ domain, in modifying SV release (von Gersdorff et al., 1997; Takahashi et al., 1998; Zamponi & Snutch; 1998).

By modulating the SV release cascade at this point, these receptors intercept the most non-linear steps in transmission, converting small local alterations in $I_{Ca}$ into much larger global ones ($I_{EPSC}$). With a tightening of VGCC-SV coupling, factors affecting the kinetics and spread of the $Ca^{2+}$ domains will be minimized on the overall $P_r$ of a SV, whereas factors impacting the probability of VGCC opening (i.e. affecting the AP shape), will have a more significant relative impact on $P_r$. A shift in release modality during development, by changing the distribution of sub-probabilities affecting $P_r$, will determine which modulating factors are most efficacious in determining the final output of the synapse. While it is clear that the nanodomain modality decreases the sensitivity of both synaptic output and delay to changes in $I_{Ca}$, it is unknown whether a release modality switch is crucial for improving the efficiency of the synapse and improving its ability to perform as a relay.

### 7.3.3 Synaptic Efficiency, Safety Factor, & Efficacy.

As it relates to single synapses, “efficiency” can be defined as the ratio of output provided by a system, versus the input required to generate it. Using this definition, and focusing on excitation/secretion coupling, we consider input to be $I_{Ca}$ with the output being the resultant $I_{EPSC}$. Of course at higher levels of processing in the CNS, the term “efficiency” may take on other interpretations where simple input-output efficacy may come at the expense of increased processing or information carrying ability (see section 7.4). Given the definition above, it is interesting to postulate which release
modality, micro- or nanodomain, provides increased efficiency to the calyx of Held-MNTB synapses and potentially to other CNS synapses.

There is some debate over whether increased efficiency is gained by employing many or few VGCCs in triggering SV release (see Stanley, 1997). Confounding this debate are issues that depend somewhat on the functional role of the synapse. It is obvious that if we consider efficiency defined as above, the nanodomain release modality is more efficient, that is, it requires less input ($I_{Ca}$) to trigger a given output ($I_{EPSC}$). However, the argument may no be so simple.

In synapses, increased efficiency is synonymous with increased sensitivity of the system, meaning that the synapse is more likely to react inappropriately to synaptic “noise” contained in the input. At a single synapse level, presynaptic noise exists as VGCC flicker while postsynaptic noise exists as mEPSCs. mEPSC may or may not be the result of presynaptic noise or VGCC flicker (Quastel et al., 1971). In this sense, the minimal unit for noise is the single channel conductance of a VGCC upon spontaneous probabilistic opening; this minimal Ca$^{2+}$ signal provides a sensitivity threshold at which release of a SV can be considered “noise”. A synapse requiring the cooperation of many VGCCs in triggering SV release is unlikely to react to spontaneous VGCC flicker resulting in lower noise. At the calyx of Held, mEPSC frequency increases with development (Joshi et al., 2004). While many of the mEPSCs are thought to be Ca$^{2+}$ independent, a significant portion, particularly in mature synapses, are the result of Ca$^{2+}$-stimulated exocytosis (MJ Fedchyshyn, unpublished observations; Lou et al., 2005). These Ca$^{2+}$-dependent mEPSCs could be due to probabilistic binding of free Ca$^{2+}$ ions in the cytoplasm, release of Ca$^{2+}$ from intracellular stores, or from spontaneous VGCC flicker in close proximity to the Ca$^{2+}$ sensor, the later being much more likely in mature synapses due to their close coupling of VGCCs and SVs (Fedchyshyn & Wang, 2005). Given the above, mature synapses with their nanodomain release modality would be inherently more noisy but also more efficient/sensitive. The issue is then whether this by-product of increased noise comes as a detriment to the functionality of the synapse.

Of primary concern is whether presynaptic noise is of sufficient magnitude to trigger a response (AP) in the output neuron (MNTB). For this to occur, mEPSCs would need to summate temporally and spatially in the case of traditional arborized synapses, to sufficiently depolarize the postsynaptic neuron and generate an aberrant AP. Even over the many AZs at the calyx of Held, and the minimal ~500pA $I_{EPSC}$ required to trigger a postsynaptic AP (Hermann et al., 2007), aberrant APs generated without presynaptic stimulation, have never been documented at the calyx of Held-MNTB synapse at resting membrane potentials. This implies that, while presynaptic noise may increase with development at this synapse, it is effectively filtered by the activation threshold of the postsynaptic neuron. In addition, this suggests that the signal-to-noise (SNR) ratio, even during periods of synaptic depression, remains quite high (Hermann et al., 2007).

A second concern regarding the availability of SVs arises when synaptic noise is increased. If too many SVs are released as noise, they may significantly reduce the number of SVs, or the readiness of the release apparatus, available for AP-triggered release. This concern is especially relevant for synapses at which the RRP is very small (i.e. hippocampal mossy fibre and cortical synapses) (reviewed in Rollenhagen & Lubke, 2006). At the calyx of Held, we have shown that in both immature and mature
synapses, even high mEPSC frequencies deplete the RRP by less than 1% (Fig. 6.12), suggesting that noise, in either type of release modality, should have a negligible effect on the number of SVs available for AP-triggered output at this synapse.

A related, but contrary, set of concerns implies that the reliability of transmission is decreased when few VGCCs are charged with releasing SVs. When SVs are released by the Ca\(^{2+}\) signal from a single VGCC, Stanley (1997) suggests that these SVs then become vulnerable to the stochastic behaviour of their associated VGCC. This follows from the fact that all voltage-sensitive ion channels open stochastically upon the appropriate voltage signal (reviewed in Neher & Stevens, 1977). On occasion, some VGCCs may not open given a presynaptic AP holding their associated SVs from fusing. In contrast, the evidence for synaptic reliability in using greater numbers of VGCCs to trigger release arose primarily from the fact that high frequency/fidelity synapses appeared to employ this modality in the CNS (i.e. calyx of Held, cochlear hair cell) (Roberts et al., 1990; Borst & Sakmann, 1996; Stanley, 1997). However, more recent accounts have shown that this is not necessarily the case. At the calyx of Held, the original estimate of ~60 VGCCs triggering release of a single SV was later reduced significantly to ~12 by the original authors in the immature synapse (Borst & Sakmann, 1996; Meinrenken et al., 2002, Meinrenken et al., 2003). Now it is clear that with maturation, this estimate decreases further again (Fedchyshyn & Wang, 2005). In the murine cochlear hair cell, Brandt et al. (2005) have found that SV release is also gated using a nanodomain modality. When these data are combined with the unexpectedly high sensitivity of the Ca\(^{2+}\) sensor (Bollmann & Sakmann, 2005), at least in the calyx of Held, it would seem that the nanodomain modality is the preferred means through which excitation-secretion coupling is gated in mature high-frequency CNS synapses. When averaged over many release sites, the odd stochastic VGCC "miss" upon AP invasion is likely to have little effect on the overall output of the synapse.

Reliability, between micro- and nanodomain modalities, hinges on different conditions within the Ca\(^{2+}\) signalling cascade. In the microdomain modality, reliability hinges on whether the AP is of sufficient amplitude and duration to ensure that a sufficient number of VGCCs open to gate release. If any one of those VGCCs fails to respond, release has a high likelihood of failure. Because VGCC cooperativity is highly non-linear in a microdomain arrangement, stochastic VGCC behaviour will have a significant impact on the P_r of any individual SV as well as its kinetics. The nanodomain modality hinges synaptic reliability on whether a single VGCC opens adjacent to a SV. In this sense release is approximately binary upon VGCC activation assuming that an individual VGCC can produce a suprathreshold Ca\(^{2+}\) signal when closely coupled to a SV. This would also suggest that SV release in this modality would be largely homogeneous in both P_r, amongst those SVs that get released, and in timing (Shahrezaei & Delaney, 2005). Since VGCC recruitment is approximately linear with presynaptic potential, ultimately the stochastic behaviour of a single VGCC will determine whether a release site is activated or not, but have a lesser effect on the overall P, of a nanodomain-dominant synapse (Furukawa et al., 1978; Furukawa et al., 1982; Brandt et al., 2005). This implies that the advantage offered by the nanodomain modality is to linearize the dependence of SV release on VGCC stochastics leading to lower single-site reliability but greater overall synaptic reliability.
Lowering site reliability can be detrimental should a synapse have a low inherent safety factor, meaning that the SV output results in a postsynaptic $I_{\text{EPSC}}$ that is only marginally larger than that required to produce an AP. At the calyx of Held, $I_{\text{EPSC}}$ regularly reach values larger than 10nA, particularly in mature synapses, suggesting that there is considerable redundancy built into this system (Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; Koike-Tani et al., 2005).

The high safety factor may serve to maintain postsynaptic APs in the face of depressing $I_{\text{EPSC}}$ during repetitive activity. Recent evidence has shown that in vivo, this synapse may be tonically depressed due to the rate of tonic AP input (Hermann et al., 2007). Thus, the extremely high safety factor may be partially an artefact of in vitro recording (i.e. lack of tonic activity), but also likely serves to ensure consistent transmission through the relay. Depressing synapses have higher release probabilities and are therefore likely to be more efficient in their use of Ca$^{2+}$ in triggering SV release (Millar et al., 2005), however once depressed, these synapses decrease markedly in their input-output efficiency and require a high safety factor to ensure postsynaptic APs are continually generated (Hermann et al., 2007). The nanodomain modality then increases input-output efficiency to the point that the minimal Ca$^{2+}$ signal can be used while maintaining a high safety factor. This further minimizes the metabolic cost of clearing large amounts of Ca$^{2+}$ ions following presynaptic activity, compartmentalizes the Ca$^{2+}$ signal, and minimizes the potential for Ca$^{2+}$-toxicity in the terminal (Stanley, 1997).

Despite the obvious efficiency advantages of the nanodomain modality, the microdomain modality also provides an effective means of releasing SVs although, given the above, it may not provide highly-efficient control of release (for review see Schneggenburger & Forsythe, 2006). What the microdomain modality does offer is highly dynamic and heterogeneous control of SV release leading to greater plasticity in synaptic timing and strength, but at the expense of true input-output efficiency. The immature synapse ensures reliable synaptic transmission by employing a wide presynaptic AP that generates a large and wide $I_{\text{Ca}}$ compared to those observed in the mature synapse (Taschenberger & von Gersdorff, 2000; Fedchyshyn & Wang, 2005). This ensures that a sufficient Ca$^{2+}$ signal is generated to trigger SV release in sufficient quantities, establishing a high safety factor for postsynaptic APs. Particularly in the immature synapse, this high safety factor is crucial for sustained transmission as postsynaptic AMPARs experience high levels of saturation and desensitization (Trussell et al., 1993; Yamashita et al., 2003; Scheuss et al., 2002; Taschenberger et al., 2002). This property of the immature synapse decreases reception efficiency and further decreases overall synaptic efficiency, especially during repetitive activity.

With maturation, the rate and extent of STD is reduced, owing somewhat to reduced desensitization and saturation of AMPARs, and $I_{\text{EPSC}}$ are more likely to remain above the threshold for generation of a postsynaptic AP. The changes in efficiency outlined above would then have implications for the filtering behaviour of the synapse, allowing the mature synapse to pass higher input frequencies for longer durations through to the LSO (Thomson, 2000; Abbott & Regehr, 2004). In contrast, immature synapses would pass shorter bursts of APs with a reduced upper limit to their frequency band. So while immature synapses are clearly more plastic in terms of their range of outputs (i.e. timing and strength,
Chapter 6), they are likely to have a reduced bandwidth and transmission sustainability compared to mature synapses.

It is unknown how these changes in the filtering properties of the relay serve to benefit higher processing functions but, given the above, it is clear that a developmental switch to the nanodomain release modality is a key step in the acquisition of highly-efficient transmission at this synapse.

7.3.4 Release Modality Switching & Development.

While it appears clear that the conversion from micro- to nanodomain release modalities is a crucial adaptation in the development of a high-fidelity synapse, a number of concurrent developmental adaptations occur around the same period (Brandt et al., 2005; Fedchyshyn & Wang, 2005, Taschenberger & von Gersdorff, 2000; Joshi & Wang, 2002; Joshi et al., 2004). Here we ask how changes in release modality fit within the broader context of development and what factors may underlie and drive the switch.

At the calyx of Held, the developmental narrowing of the presynaptic AP appears to be offset by the tighter coupling and increased efficacy offered by the nanodomain release modality (Taschenberger & von Gersdorff, 2000; Fedchyshyn & Wang, 2005; Yang & Wang, 2006). This is observable in the relative consistency of afferent evoked IEPSC amplitudes across development in rats (Chuma & Ohmori, 1998; Taschenberger & von Gersdorff, 2000) and an increase in their amplitudes across development in mice (Joshi & Wang, 2002; see also Yang & Wang, 2006). Despite significant decreases to the magnitude of Ica, mature synapses maintain their output strength and inherit increased input-output efficiency (see section 7.3.3). However, besides increased efficiency, there are other advantages to this seemingly redundant set of adaptations.

By narrowing AP width, through the presynaptic expression of more rapid Na+ and K+ channels, mature calyces are able to cycle their membrane potentials more rapidly thereby increasing the upper limit for one-to-one AP reproduction, expanding their dynamic range (Leão et al., 2005; Nakamura & Takahashi, 2007). As discussed above, morphological changes that increase glutamate clearance rates and changes to the AMPAR subunits decrease AMPAR saturation and desensitization allowing for the newly increased bandwidth to be faithfully propagated. In addition, loss of NMDARs can aid in the synchronous reproduction of input signals by decreasing the postsynaptic plateau potential observed during high frequency postsynaptic AP firing (Joshi & Wang, 2002; Joshi et al., 2007). However, the synapses ability to rapidly generate and receive APs hinges on the fact that upstream a smaller Ca2+ signal can be effectively sensed by the release machinery, hence the requirement for a nanodomain coupling modality.

SD also decreases with maturity, downstream of Ica activation, due most likely to a decrease in Ca2+ ion transit time/distance and a sharper Ca2+ profile experienced at the Ca2+ sensor. In Chapter 5 we showed that larger Ca2+ signals propagate with shorter SD in both immature and mature synapses. During development, the VGCCs underlying SV release switch from a mix of N and P/Q-types to exclusively P/Q-type (Iwasaki & Takahashi, 1998; Ishikawa et al., 2005). P/Q-type VGCCs have been found to specifically underlie the phenomenon of activity-dependent Ica facilitation at the calyx of Held
Their exclusive presence at the mature synapse may serve to amplify $I_{Ca}$ during repetitive activity, such that SD is further reduced and a greater safety factor is achieved. A decrease in SD could present a survival advantage in a sensory relay such as the calyx of Held-MNTB synapse, where minimal delay would allow for the most rapid and accurate localization of sounds in three-dimensional space. By minimizing the $Ca^{2+}$ signal necessary for SV release, the mature synapses also minimizes the build-up of $[Ca^{2+}]$, which has been shown to underlie activity-dependent prolongation of SD thereby maintaining the most consistent AP timing possible (Fedchyshyn & Wang, 2007).

Certainly, other developmental changes may occur at the calyx of Held, whose interplay with developmental release modality tightening are yet to be elucidated. Mobile buffers within the terminal may change in type or concentration with development, shaping $Ca^{2+}$ domains differently, impacting $P_r$ and thus the kinetics and strength of SV release (see Burnashev & Rozov, 2005). However, by using the whole-cell recording configuration in our experiments, these changes are controlled for as most mobile buffer would be dialyzed rapidly with that of the recording pipette. Recently, parvalbumin was identified as a mobile $Ca^{2+}$ buffer in the immature calyx of Held, which aids in the establishment of accurate model parameters describing the shaping of $Ca^{2+}$ domains by buffers within the terminal (Müller et al., 2007). Unfortunately, little is known about similar parameters in the mature synapse (>P13-14) leading to some unsubstantiated generalization based on immature synapses. Future experimentation and modelling studies should focus on distinguishing the differences in presynaptic composition between immature and mature synapses with buffer make-up being a key target.

An alternative approach to understanding how developmental adaptations at this synapse coalesce is to elucidate the mechanisms underlying their expression. What triggers a tightening in release modality? Interestingly, we observe a loss of N-type VGCCs and a tightening of the remaining P/Q-type VGCCs around the onset of hearing (~P11) (Fedchyshyn & Wang, 2005). While this does not exclude the possibility that this transition could be the manifestation of genetic programming unrelated, but coincident, to the opening of the ear canal, it provides a compelling path on which to investigate activity-dependent remodelling of the synapse. Using genetically deaf mice, and experiments similar to those in Chapter 3, we could determine whether a tightening of the release modality is delayed, or occurs at all, in the absence of auditory input. Alternatively, cochlear ablation could be used to eliminate input into the contralateral nucleus within the brainstem while maintaining input to the ipsilateral side establishing an in-slice control system. Experiments such as these, while coarse, could suggest whether activity drives this subsynaptic change. Delving further, we could ask whether $Ca^{2+}$ driven by activity, or an unidentified signalling molecule, initiates the molecular cascade defining the positioning of certain VGCC subtypes.

Some preliminary evidence exists to suggest that VGCCs may occupy a set of existing “slots” near SVs which may be preferential for N or P/Q-type VGCCs. The molecular make-up of these “slots” may change with development refining their preference to favour solely the P/Q-type VGCCs and thereby tightening their coupling with the release machinery (Cão et al., 2004). However, to confirm this hypothesis, a much better understanding of the protein composition of the AZ within both the immature
and mature calyx will have to be achieved. Unfortunately, due to the relatively low density of calyces within the MNTB, quantitative molecular biology has proven difficult (Joshi et al., 2004). In addition, the lack of success in generating viable culture of the calyx of Held-MNTB synapse has restricted the highest resolution imaging from this system.

Alternatively, as suggested herein (Chapter 5), the SVs themselves may couple more tightly with the membrane and thus the VGCCs. Here we have implicated Sept5 as defining the “vertical” separation of SVs from the AZ membrane and have provided functional data to support TEM images (Chapter 5 & 6). However, it remains unknown what role this protein assumes later in development after SVs have coupled tightly to AZ membranes. More convincing evidence implicating Sept5 in SV localization would come from immuno-gold labelling of the protein and direct imaging of its presence in the space between SVs and the AZ in immature terminals. Repeating these experiments in mature synapses may also hint as to what functions this protein may be serving later in development. We are currently attempting such experiments. Since its most likely SNARE binding partner is syntaxin, presynaptic loading of botulinum toxin C1 may hint as to whether Sept5 is interacting with syntaxin containing SNARE complexes in the manner hypothesized in Chapter 5 (Beites et al., 1999; Beites et al., 2005; Meunier et al., 2002). In any case, determining the mechanism by which a tightening in the relationship between VGCCs and SVs occurs will provide better understanding of how a single synapse remodels with development and acquires a high-fidelity character. Moreover, it may allow us to better understand why the microdomain modality develops in the first place.

From our findings in Chapter 5 & 6, it is clear that the use of many VGCCs in triggering SV release provides the synapse with a greater dynamic range in synaptic output, greater heterogeneity in \( P_b \), and a wider variety of STP patterns. However, it is not understood why these are desirable characteristics for a developing synapse of this type. These characteristics come at the cost of efficiency, reliability, rapid release kinetics, frequency response range, and minimization of \([Ca^{2+}]_i\). It seems much easier to rationalize the nanodomain modality for high-frequency synapses than it does to envision a distinct advantage for use of microdomains.

A few possibilities exist for why microdomains may be used early in synaptic development at the calyx of Held. Distant VGCC-SV spacing may function in an “adaptive” role to maintain reasonable synaptic output given wide APs (Leão et al., 2005; Nakamura & Takahashi, 2007). However, this assumes a deterministic role in synaptic maturation for presynaptic \( Na^+ \) and \( K^+ \) channels for which there is no current evidence. For example, similar rationale could be applied should release modality switching be genetically programmed while changes in presynaptic \( Na^+ \) and \( K^+ \) channels evolved to compensate. Interestingly, expression of \( K_{v3} \) channels increases during postnatal development in many regions of the rodent CNS, while \( K_{v1} \) channels increase in density in culture also, raising the possibility that their change in expression may be programmed (Perney et al., 1992; Gurantz et al., 2000; Nakamura & Takahashi, 2007). In either case, the causal role for these changes remains unknown.

It is possible that our observations at the immature synapse are indicative of a “generic” synapse which contains all of the ingredients for formation of a “specialized” one but lacks the signal to initiate its consolidation.
An alternative formulation of this hypothesis is that the synapse is still in the process of formation; however this is unlikely given that functional synapses can be observed as early as P1-2, suggesting that their formation occurs much earlier than P8 when our earliest recordings are performed (Hoffpauir et al., 2006). In this case, the “generic” synapse would require some form of activation signal to trigger its specialization which could come in the form of activity or from any number of neuronal or glial signalling mechanisms (reviewed in Craig et al., 2006). Since MNTB neurons are arranged in a tonotopic order within the nucleus, this could be a mechanism by which fine-tuning of each synapse is accomplished given a certain input, analogous to various forms of presynaptic LTP (Pelkey et al., 2006; Lauri et al., 2007). Interestingly, the establishment of the tonotopic arrangement of K⁺ channels appears to be dependent on spontaneous activity during development, which is absent from congenitally deaf mice (Brew & Forsythe, 2005; Leão et al., 2006). Thus the fine-tuning of this K⁺ current gradient by activity provides compelling evidence that at least some fundamental properties of the synapse are specific to certain forms of activity.

While there is no current evidence to suggest that there may also be a parallel gradient of cooperativity (m) values, changes in input, amplified by the high m-value in immature synapses, could serve to rapidly engage any number of signalling cascades. The highly variable I_{EPSC} amplitude, given the high m-value, could serve to amplify small differences in presynaptic input into large differences in output. Given the number of retrograde signalling pathways identified at the calyx of Held and other CNS neurons (i.e. endocannabinoids, NO, CO, arachidonic acid, neurotrophins, BDNF, mGluRs), it is possible that increased activity in the postsynaptic neuron could affect VGCCs presynaptically (Kushmerick et al., 2004; reviewed in Fitzsimonds & Poo, 1998).

Given the possibilities above, the mechanisms underlying the conversion of VGCC-SV coupling from micro- to nanodomain modalities provides an intriguing target for future studies. While beyond the scope of this thesis, the calyx of Held provides a unique and accessible experimental tool for increasing our understanding of synaptic maturation and specialization. Identifying what drives the rapid specialization of this synapse may also aid in our understanding of why these changes are necessary for reliable transmission of high frequency signals.

### 7.3.5 Mechanisms Underlying STP at the Calyx of Held & Release Modality Switching.

The short-term modulation of signals through synapses is the fundamental processing unit in the CNS. Even a synapse as “simple” or “rigid” in purpose as the calyx of Held-MNTB synapse exhibits significant signal modulation when input is altered. Inputs arrive to this synapse in trains of APs which range in frequency and are variable in their patterns of output (I_{EPSC}) as a result (Hermann et al., 2007). We have investigated how the patterns of STP in both synaptic strength and timing are dependent on the release modality of the synapse (see Chapter 6). Below we discuss how this knowledge compliments or contradicts the current understanding of STP at the calyx of Held.

STD is generally thought to arise from the depletion of release competent SVs, although opposing explanations have been put forth (Weis et al., 1999; Hsu et al., 1996). At the calyx of Held, activation of presynaptic mGluRs (von Gersdorff et al., 1997), AMPAR saturation and desensitization
(Scheuss et al., 2002; Otis et al., 1996), depletion of Ca\(^{2+}\) ions from the synaptic cleft (Borst & Sakmann, 1999) and VGCC inactivation at low frequencies (Forsythe et al., 1998) have all been implicated in the decreasing I\(_{\text{EPSC}}\) observed in response to AP trains. The extent to which any of these mechanisms impact I\(_{\text{EPSC}}\) may also depend on the developmental stage of the synapse (Taschenberger & von Gersdorff, 2000; von Gersdorff & Borst, 2002; Taschenberger et al., 2005).

In our experiments we found that synapses with low m-values (nanodomain) consistently showed greater STD than did synapses with high m-values (microdomain) for a given AP waveform (Fig. 6.1). In these experiments, presynaptic AP frequency was well above that at which VGCC inactivation is observed (Forsythe et al., 1998). [Ca\(^{2+}\)] in the synaptic cleft appears to also be stable as I\(_{\text{Ca}}\) maintained a relatively constant amplitude throughout the AP trains (Fig. 6.1). In addition, mGluR activation has been shown to have a very small impact on synaptic output and not to affect STP at higher frequencies at all (von Gersdorff et al., 1997; Billups et al., 2005). Only saturation and desensitization of AMPARs appears to be a significant contributor to STD at this synapse under the experimental conditions presented herein. However, the contribution of AMPAR desensitization likely causes overestimation of the actual extent of STD especially in immature synapses (Joshi et al., 2004; Taschenberger et al., 2005), suggesting that the differences observed between immature and mature synapses here are most likely underestimated. We were also able to exclude Ca\(^{2+}\)-dependent release machinery exhaustion (Stevens & Wang, 1995; Hsu et al., 1996; Dobrunz et al., 1997; Dittman & Regehr, 1998; Dittman et al., 2000) as a cause of STD and activity-dependent increases in SD (Fig. 6.13, 6.14).

Interestingly, we found that activity-dependent increases in SD correlate with depletion of the RRP, with the severity of the increase in SD being dependent on the build-up of [Ca\(^{2+}\)] (Fedchyshyn & Wang, 2007). This correlation was strengthened by the observation that increases in SD recovered with similar timescales as the RRP did from depletion (Fig. 4.9). From these data, it appears that, the more rapidly the RRP is utilized, the slower the kinetics of SV release become. The rate at which the RRP is used depends directly on the P, of the synapse which depends strongly on the coupling of VGCCs and SV for a given AP waveform. When tightly-coupled SVs are used rapidly, either through rapid increases in [Ca\(^{2+}\)], or tight VGCC-SV coupling, recruitment of distant SVs and the increased transit time for Ca\(^{2+}\) ions to reach them is measurable as a consistent increase in SD. Simultaneously, the rapid use of tightly-coupled SVs renders those AZs temporarily void of releasable SVs resulting in rapid decreases in quantal output during trains. By altering the P, at individual AZs, the coupling between VGCCs and SVs can determine the rate at which the RRP is depleted and thus the kinetics of STD and correlated increases in SD. With development, the increased P, is managed primarily by decreasing the number of AZs which are recruited for any given AP through the narrowing of the AP waveform (Taschenberger & von Gersdorff, 2000; Fedchyshyn & Wang, 2005). In addition, it should be noted that the RRP size increases significantly with development (Taschenberger & von Gersdorff, 2000) and that more SVs appear physically docked at the AZs (Chapter 5) which may aid in minimizing increases in SD and the rate of STD in mature synapses. In contrast, immature, or high-m, synapses minimize P, downstream of I\(_{\text{Ca}}\) by requiring the cooperation of many VGCCs which aids in maintaining the RRP for sustained
transmission. Interestingly, and particularly when AP width is controlled, this strategy for $P_r$ reduction can also result in STF.

The mechanisms underlying STF are somewhat more intriguing and controversial than those of STD. Facilitation depends not only on whether an appropriate signal is present, but also on whether a SV is present to be facilitated. In this way STF also depends directly on whether and to what extent STD has occurred. One of the most elegant and simple illustrations of this concept was put forth in a model for STP proposed by Dittman et al. (2000). In this model, all basic forms of STP could be explained by mixing; a factor increasing $P_r$ (Ca$^{2+}$-dependent facilitation of release), a factor decreasing $P_r$ (AZ unavailability), and a factor dictating the replenishment rate of SVs (Ca$^{2+}$-dependent replenishment). AZ unavailability could arise from a number of sources, however, as above; it appears that this is due primarily to RRP depletion at the calyx of Held (Chapter 6). Using different proportions of these components, Dittman et al. (2000) could reproduce various forms of STP including the sequential STF/STD observed at the immature calyx of Held under conditions of low $P_r$ (Chapter 6; Taschenberger et al., 2005).

A number of mechanisms have been proposed to account for STF with most being variations on the “residual Ca$^{2+}$ hypothesis” originally proposed by Katz and Miledi (1968). The mechanistic debate concerning the residual Ca$^{2+}$ hypothesis revolves more around how Ca$^{2+}$ ions act to increase $P_r$, than whether elevations in [Ca$^{2+}$] is actually the underlying cause (reviewed in Zucker, 1999; Zucker & Regehr, 2003). The simplest and original formulation of the residual Ca$^{2+}$ hypothesis assumes that Ca$^{2+}$, left over from previous events, adds linearly with Ca$^{2+}$ from upcoming events to increase $P_r$. Much debate centers around why, with more current understanding of the relationship between Ca$^{2+}$ and SV release, the linear addition of [Ca$^{2+}$], and domain [Ca$^{2+}$] do not completely account for the observed increase in synaptic output (Zucker & Regehr, 2002). To account for the mild (~1.4 fold) non linearity in the addition of domain Ca$^{2+}$ and [Ca$^{2+}$], a high-affinity Ca$^{2+}$ binding site has been proposed (Yamada & Zucker, 1992; Felmy et al., 2003). Debate as to whether this “facilitation site” is part of the release machinery (Yamada & Zucker, 1992; Bertram et al., 1996) or a completely separate entity (Atluri & Regehr, 1996; Dittman et al., 2000; Matveev et al., 2002) continues. In either formulation, it is assumed that binding of Ca$^{2+}$ to this site, increases the $P_r$ of the sensor to subsequent VGCC openings. These facilitation sites were necessary to explain why such a relatively small addition to domain Ca$^{2+}$ could generate such large increases in $P_r$. A by-product of this was that the Ca$^{2+}$ sensor must be of relatively low-affinity in order to explain why it was not saturated given domain Ca$^{2+}$ of an estimated 100μM and the steep dependence of release on $I_{Ca}$ (Dodge & Rahamimoff, 1967; Simon & Llinas, 1985; Yamada & Zucker, 1992).

More recent evidence from the Calyx of Held has forced revisiting the assumptions of these models. The domain Ca$^{2+}$ signal estimated from the calyx of held has been found to be closer to 10μM (Bollmann et al., 2000) and the sensitivity of the Ca$^{2+}$ sensor is accordingly much higher than originally estimated (Bollmann et al., 2000) meaning that it is capable of monitoring much smaller changes in [Ca$^{2+}$] than originally thought.
A subsequent study by Felmy et al. (2003) adds a series of crucial pieces to the understanding of Ca\(^{2+}\)-dependent facilitation. First, the linear summation of domain and residual Ca\(^{2+}\) does account for a measurable portion of STF. Second, facilitation is mediated exclusively by processes downstream of Ca\(^{2+}\) entry. Third, the decay of facilitation correlates with the decay of [Ca\(^{2+}\)]. Lastly, facilitation does not appear to measurably change the sensitivity of the release machinery or the kinetics of SV release implying that it is generated by increases to the [Ca\(^{2+}\)] experienced by the Ca\(^{2+}\)-sensor. As a result of this final observation, Felmy et al. (2003) hypothesize that the unexplained non-linearity in [Ca\(^{2+}\)] summation is the result of partial saturation of Ca\(^{2+}\) buffers in the vicinity of SVs (see also Neher, 1998).

This is in contrast to our findings in which we observe a measurable decrease in SD when a synapse is primed using mild depolarizing steps (Fig. 6.13), suggesting that the sensitivity of those SVs is increased. In addition, we measure decreases in SD during trains in which STF is present (Fig. 6.5). This discrepancy could be the result of our use of a highly accurate and unbiased method of SD measurement (Fedchyshyn & Wang, 2007) or due to an artefact of the kinetic limitations of the Ca\(^{2+}\) uncaging technique (see Felmy et al., 2003). In the framework of Felmy et al. (2003), our results suggest that perhaps a “bound model” of facilitation could account for the non-linear addition of [Ca\(^{2+}\)] at the calyx of Held. However, our interpretations on the role of release modality in determining the characteristics of STF are applicable to either a buffer saturation or bound model of STF.

At the calyx of Held, part of STF can be attributed to facilitation of I\(_{\text{Ca}}\) (Cuttle et al., 1998; Tsujimoto et al., 2002) and part to steps downstream of Ca\(^{2+}\) influx (see above). I\(_{\text{Ca}}\) facilitation appears to be a property of P/Q-type VGCCs exclusively (Inchauspe et al., 2004; Ishikawa et al., 2005), although in both age groups tested here I\(_{\text{Ca}}\) facilitation appeared similar in magnitude (Chapter 6). This suggests that the proportion of P/Q-type VGCCs in the immature synapse is significant enough to generate significant increases in I\(_{\text{Ca}}\) during AP trains. In addition, it appears that a portion of I\(_{\text{Ca}}\) facilitation results from an appropriate inter-AP-interval, while the rest relies on the build-up of [Ca\(^{2+}\)], possibly binding to NCS-1 (Tsujimoto et al., 2002).

While it is clear that an increase in I\(_{\text{Ca}}\) will have a profound effect on SV release, we were able to compensate for I\(_{\text{Ca}}\) facilitation to investigate exclusively those processes, downstream of I\(_{\text{Ca}}\), which culminate in I\(_{\text{EPSC}}\) facilitation. Interestingly, only immature (high-m) synapses exhibit I\(_{\text{EPSC}}\) facilitation under these conditions (Fig. 6.14) suggesting that a larger distance between SVs and VGCCs is required for the expression of I\(_{\text{EPSC}}\) facilitation. This can be rationalized by considering the position of the Ca\(^{2+}\) source relative to the SV.

As above, expression of facilitation requires both the facilitation signal (Ca\(^{2+}\)) and the presence of a SV with associated release machinery to receive that signal. When the distance between VGCCs and SVs is large, the opening of VGCCs is more likely to generate sub-threshold Ca\(^{2+}\) signals in the vicinity of the SV. This transient sub-threshold increase in [Ca\(^{2+}\)], may result in incomplete occupancy of the Ca\(^{2+}\) sensor or partial saturation of mobile buffers (Neher, 1998) which result in a transient decrease in buffer capacity and an increase in P, at that SV. In fact, mobile buffers shape the size and spread of Ca\(^{2+}\) domains within the nerve terminal and thus will determine how many Ca\(^{2+}\) ions arrive at the binding sites on the Ca\(^{2+}\) sensor (Helmchen et al., 1997; Naraghi & Neher, 1997; Meinrenken et al., 2002; Müller
et al., 2007). In the case of a bound model, \( P_r \) is increased with partial occupancy, meaning that fewer \( \text{Ca}^{2+} \) ions must bind to the \( \text{Ca}^{2+} \) sensor, upon subsequent VGCC openings, for release to be triggered. So separation and interpretation of the role these two mechanisms play in facilitation may not be straightforward. However, the key factor here is that the SV and VGCC are separated by a significant distance which, in the context of a bound model allows for domain \( [\text{Ca}^{2+}] \) to degrade before reaching the \( \text{Ca}^{2+} \) sensor, and in the context of a buffer saturation model allows sufficient time for \( \text{Ca}^{2+} \) to interact with the buffer (i.e. distances longer than the buffer length constant) (Naraghi & Neher, 1997). In our experiments, the whole-cell recording arrangement most likely results in dialysis of endogenous mobile buffers, replacing them with those contained in the pipette (0.5/10mM EGTA). Thus, the potential for developmental changes in endogenous buffer composition affecting our interpretation of the data are probably minimal (Felmy & Schneggenburger, 2004).

From the above, we can see that the microdomain release modality provides the necessary physical ingredients to allow for facilitation of transmitter release through either bound or buffer saturation models. In particular, the recent elucidation of the slow buffer parvalbumin as one of the endogenous presynaptic \( \text{Ca}^{2+} \) buffers at the calyx of Held highlights the importance of VGCC-SV separation in the buffer model (Müller et al., 2007).

In contrast, tightly-coupled nanodomain coupling of VGCCs and SVs change the effectiveness of both of the facilitation models outlined here. Although the exact VGCC-SV separation distance at the mature calyx is unknown, estimations from other systems which exhibit similar cooperativity \( m \) and patterns of short-term plasticity can be as little as 20nm (Heuser et al., 1974; Pumplin & Reese, 1978; Gulley et al., 1978; Pumplin et al., 1981; Harlow et al., 2001; Stanley et al., 2003) as opposed to the \( \approx 100 \text{nm} \) estimated at the immature calyx of Held (Meinrenken et al., 2002). A change in coupling of this magnitude has significant implications for the way in which SVs are released. Upon VGCC opening, the domain \( [\text{Ca}^{2+}] \) experienced by the \( \text{Ca}^{2+} \) sensor will be significantly elevated as compared to VGCCs further away due to diffusion and the inability of slow buffers to interact with the \( \text{Ca}^{2+} \) signal (Naraghi & Neher, 1997). Thus given the high-affinity of the \( \text{Ca}^{2+} \) sensor (Bollmann et al., 2000), it is likely that upon channel opening, the \( \text{Ca}^{2+} \) binding criterion is rapidly satisfied in this modality by one or a few VGCCs (Shahrezaei et al., 2006). Thus, the probability that a SV will experience sub-threshold \( \text{Ca}^{2+} \) signals is very low. So while \( [\text{Ca}^{2+}] \) may in fact be elevated, and/or endogenous buffers may be partially saturated following an AP, the SVs in the vicinity of these elevations will have been released and thus are not available for subsequent facilitated release.

The differences in excitation-secretion coupling dictated by the release modality can be observed in high-frequency trains of APs. When AP waveform is controlled, mature synapses exhibit simple and consistent STD in all but the lowest \( P_r \) conditions (i.e. very narrow APs in low \([\text{Ca}^{2+}]_o\)). This STD is caused by the lack of a facilitation component (as above) and progressive depletion of available SVs. In contrast, immature synapses show significant facilitation early in trains followed by STD as SVs become depleted and eliminate any facilitated SVs (Fig. 6.1). However, the fact that some SVs release, others facilitate, and yet other do not release at all, suggest that the microdomain release modality also imparts heterogeneity across SVs. Indeed, heterogeneity in \( P_r \) has been a consistent requirement to explain the
observed forms of STP generated from immature calyces (Meinrenken et al., 2002; Sakaba et al., 2002; Trommershäuser et al., 2003; Wadel et al., 2007). Recent evidence suggests that this heterogeneity may arise from a “positional heterogeneity” in the coupling of VGCCs and SVs (Trommershäuser et al., 2003; Wadel et al., 2007). When we consider the possible range of $[\text{Ca}^{2+}]$, generated due to the requirement for many VGCCs in triggering release and the stochastic nature of their opening upon AP innervation, heterogeneity in $P_r$ follows easily.

In the mature synapse, we expect release to depend almost solely on whether an AP activates an associated VGCC, as opposed to also depending on the occurrence of a significant degree of VGCC cooperation. Thus, $P_r$ would approach a near binary quantity dependent only on the stochastic nature of the VGCC opening. The consequence of this is that the degree of heterogeneity in $P_r$ across SVs would approach zero. Under these conditions we would expect relatively consistent patterns of STP despite changes to the input (APs), which is what we observe from mature synapses (Chapter 6). Pushing the limits of the experimental window at the calyx of Held past P18 may yield even further consolidation of the patterns of STP. It should be noted that, despite a nanodomain arrangement, heterogeneity in $P_r$ can arise simply from the positioning of the $\text{Ca}^{2+}$ sensor relative to the $\text{Ca}^{2+}$ source (Shahrezaei & Delaney, 2005) so the above represents a somewhat “stripped-down” interpretation of the data presented herein.

Given the above, it appears clear that the position of VGCCs relative to SVs, in concert with the resultant buffer dynamics and the diffusion of $\text{Ca}^{2+}$, plays a crucial role in determining the patterns of STP and release kinetics observed at this and potentially other synapses.

SECTION 7.4: IMPLICATIONS OF RELEASE MODALITY TIGHTENING ON SYNAPTIC SIGNAL PROCESSING

7.4.1 The Sound Localization Circuit & the Calyx of Held-MNTB Synapse.

The calyx of Held-MNTB synapse is a unique in that it that allows for unprecedented resolution in the study of fundamental synaptic transmission in the CNS. However, this synapse is part of a larger network or synapses which serve to localize sound in the horizontal plane (azimuth) (reviewed in Oertel, 1991; Oertel, 1997; Oertel, 1999). One branch of the network appear to localize differences in phase between the two ears, useful only for low frequency sounds that have wavelengths longer that twice the thickness of the animals head. For these sounds, differences in the wave-front encountered at each ear are termed interaural timing differences (ITDs). The other branch seems tuned to use differences in sound pressure level (SPL) or “volume” between the two ears to place code the source. This method of sound localization is called interaural level difference (ILD) and discriminates the location of high-frequency sounds with a wavelength shorter than twice the thickness of the animals head. While these two circuits have frequency-segregated recruitment, the level of cross-talk and interconnectivity between the two is not well understood (Oertel, 1999). In this light, we will describe these circuits in their minimal form and speculate on how a change in release modality may improve the ability of the network to effectively localize sound in space.
In rodents, integration of the signals involved in discriminating ITDs occurs in the medial superior olivary nucleus (MSO) while the same task occurs in the lateral superior olivary nucleus (LSO) for ILDs (Smith et al., 2000). Both of these centers receive excitatory input from the ipsilateral spherical bushy cells (SBCs) of the anteroventral cochlear nucleus (aVCN) while the MSO also receives excitatory input from the contralateral SBCs (Smith et al., 2000). Also located within the aVCN, the globular bushy cells (GBCs) project excitatory inputs across the midline through their giant calyceal nerve terminals (the calyx of Held) to the principle cells of the MNTB. MNTB neurons then act as a sign inverting relay and project an inhibitory signal, via primarily glycine but also GABA_A to both the ipsilateral MSO and LSO (Spangler et al., 1985; Smith et al., 2000). At both of these centres, coincident excitatory and inhibitory inputs are initially integrated into a signal, coding the location of sound, which is sent to higher CNS nuclei (inferior colliculus, IC, and dorsal nucleus of lateral lemniscus, DLL) for further processing (Glendenning et al., 1992).

The system of coincidence detection is highly accurate, depending somewhat on the species and on the separation between the ears. Owls are capable of determining the location of a sound in space to within 1.5° which requires separation of interaural timing differences of ~1.5μs (Knudsen & Konishi, 1979; Carr, 1993). The echolocation system of bats is even further tuned to detect interaural jitter in the arrival of sound waves of as little as 10ns (Simmons et al., 1990). While these particular examples are exceptionally well evolved for this purpose, most other species are still able to discriminate phase differences in the low μs range which illustrates the requirement for highly accurate signal transduction and integration throughout the circuit. Preservation of timing is crucial for both ITD and ILD as all auditory signals are essentially transduced into a frequency signal or temporal code before integration by their respective coincidence detector (Sanes, 1990). This is accomplished through phase locking, the temporal coincidence of AP generation with the peaks of sound wave stimuli, at the GBC/SBCs.

The effectiveness of phase locking depends on the membrane properties of the synapses being able to keep up with the phase frequency of the input signal, but also depends on the intensity of the input signal which becomes more effective at triggering coincident APs with increasing amplitude (Paolini et al., 2001; Cook et al., 2003). When the phase frequency is too rapid for AP generation on every wave peak, phase-locking neurons will fire on only a fraction of peaks (i.e. every other or every third peak) meaning that the representation of sound frequencies and placement is coded at firing rates significantly lower than that of the actual tone (Cook et al., 2003). Given the above, it has been suggested that the ability to discriminate phase differences, for low-frequency sounds, has evolved as a by-product of the development of the ILD system (Joris & Yin, 1998; Oertel, 1999).

The Calyx of Held-MNTB synapse appears ideally suited for reliable high-frequency transmission owing to multiple morphological and functional adaptations (see Chapter 1). These characteristics and the observance of robust short-term plasticity, primarily STD, make it an intriguing and accessible target system for understanding the role of STP in auditory processing. Furthermore, the developmental changes occurring around the time of hearing onset can provide functional clues as to what synaptic properties are crucial for the refinement of sound localization (Taschenberger & von Gersdorff, 2000;
The obvious uniqueness of this synapse lies in its morphologically specialized calyceal presynaptic terminal. This structure essentially places the many synapses normally distributed across conventional arborized neurons, into a single, relatively compact structure. In this sense, each AZ within the calyx of Held is analogous to a more typical “bouton” synapses with the key difference that, upon AP innervation, each of these “synapses” will experience consistent voltage oscillations. This consistency is due in part to the heminodal location and high density of Na$^+$ channels which amplify and distribute APs evenly throughout the terminal (Leão et al., 2005). This positioning provides an advantage in terms of synaptic reliability as each AZ can maintain a relatively low P$_{BR}$ while ensuing activation of a sufficient number of AZs to generate APs in the postsynaptic MNTB neuron (Lisman, 1997; Oertel, 1997).

As above, APs generated in the calyx of Held arise from GBCs which are similar to MNTB neurons in that they project large thick axons, have highly non-linear I-V relationships, short membrane time constants, and receive excitatory input through calyceal nerve terminals, the endbulb of Held from the 8$^{th}$ auditory nerve in the case of the SBC (Morest, 1968; Oertel, 1999). This allows the inhibitory portion of this network to bypass the need for precise spatiotemporal coordination of many smaller inputs in the generation of APs to the next relay (Oertel, 1997). Moreover, these adaptations allow for the transmission of inhibitory/contralateral information to the LSO with delay times on the order of those of the excitatory/ipsilateral portion of the circuit despite the significantly longer transmission distance and extra synapse (Boudreau & Tsuchitani, 1968; Tsuchitani, 1988). Interestingly, responses measured at the MNTB are nearly identical to those generated at the GBC, suggesting a circuit tuned to reproduce signals generated in the hair cells of the auditory nucleus (Joris & Yin, 1998). In the rat, APs transmitted through the auditory nerve to the GBCs are generally phase locked to oscillations of the hair cells at frequencies below 1500Hz, although it appears that the upper limit for maintenance of vector strength is closer to 800Hz (Paolini et al., 2001). This is in line with the upper estimates for faithful reproduction of APs at the calyx of Held-MNTB synapses at ~1000Hz (Taschenberger & von Gersdorff, 2000; Wu & Kelly, 1993). This suggests that both of these synapses are idealized for transmission of APs below 1000Hz and that the prevalence of phase locking is most likely determined in the GBC with the MNTB apparently designed for their reproduction (Paolini et al., 2001).

7.4.2 Coincidence Detection.

Coincidence detectors receive one or more inputs and produce an integrated output based on the coincidence of input timing. In the auditory brainstem, this can be most easily observed in the nucleus laminaris (NL) of the chick which receives and integrates both ipsilateral and contralateral excitatory inputs. These inputs arrive coincidentally, summating and producing robust activity in the NL when sound is coincident at each ear (Cook et al., 2003). When these signals are out of phase, called “phase delay”, the firing rate of the NL neuron decreases as neither ipsilateral nor contralateral excitatory input alone is capable of driving significant activity in the target neuron (Cook et al., 2003). The MSO, in rodents for example, functions in an analogous way to the NL of the chick in that it also receives binaural excitatory input (Carr, 1993; Oertel, 1999; Cook et al., 2003). In this sense, the precise timing of APs
becomes of paramount importance. Key to this model is the concept of “delay lines” which impose phase delays across the ears depending on their respective activation (Jeffress, 1968). Because of the tonotopic arrangement of most nuclei in the auditory brainstem, recruitment of different synapses with different dendritic path lengths or different membrane properties is thought to establish the phase differences that are required for the localization of sound in this model (Joris et al., 1998; Thompson, 2003). Reliable coincidence detection hinges on the ability of the integrating neurons to maintain the ability to discriminate input phase differences, independent of the input frequency, without saturation of its spiking ability (Cook et al., 2003). Any means of altering any of the input signals (i.e. feedforward, feedback, or bidirectional modulation), enhances the computational potential of each neuron by adjusting its response timing (Abbott & Regehr, 2004). Further downstream, the size of the EPSP/IPSPs can alter the timing of arriving inputs, with larger events occurring with the shortest latencies at the LSO (Sanes, 1990). Indeed we confirmed that latency (SD) across the calyx of Held-MNTB synapses can be minimized by employing tight coupling of VGCCs and SVs in combination with large I_{Ca} (Chapter 5). Thus when considering temporal coincidence we must also consider the temporal effects of modulating synaptic strength upstream especially when source timing must be discriminated on the order of µs (Park et al., 1997).

However, despite the simplicity of the Jeffress model of coincidence detection (Jeffress, 1948; Jeffress, 1968), most coincident detectors also receive inhibitory inputs making the interpretation of their decoding strategy difficult.

7.4.3 The Role of Inhibition in Coincidence Detection.

Accounting for inhibition, in treating the sound localization circuits as coincidence detectors, adds a new level of complexity when determining a given neurons function within the network. While it is easy to visualize the addition of temporally synchronized inputs exciting a recipient neuron and coding for sound placement, the purpose of coincident inhibition may be more subtle. In the ITD circuit, there is evidence from the gerbil, guinea pig, chick, and bat that the response window, the set of phase differences that a coincidence detector will respond to, is refined when inhibition is present (Grothe & Sanes, 1994; Grothe & Park, 1998). In these systems, inhibition is usually slightly out of phase with excitation. Thus IPSPs would shunt the excitatory response only when they are temporally unmatched serving to increase discrimination between coincident and slightly out of phase inputs (Grothe & Sanes, 1994; Funabiki et al., 1998). In this arrangement, inhibition can act as physiological “delay lines” in the Jeffress model by creating the phase limits for response (Jeffress, 1968).

Grothe & Park (1998) found that certain neurons of the MSO, do not exhibit Jeffress-like coincidence detection behaviour due to the fact that monaural excitation was capable of driving significant activity independent of phase angle. When inhibition was observed, symmetric and robust coincidence detection was also generally observed. This suggests that inhibition may create artificial delays in neurons which are particularly excitable, establishing their response window. Moreover, it may refine the phase detection threshold of neurons with existing response windows especially for animals with small heads and short distances between ears.
Alternatively, inhibition may serve as a means of gain control (Funabiki et al., 1998). As described above, changes to sound intensity (SPL) or to sound frequency can change the input pattern to the MSO/LSO. Coincidence detectors must maintain their ability to convert these input signals into meaningful information regarding the placement of the source, independent of the level or frequency (Cook et al., 2003). Inhibition may then act to decrease the output rate of MSO/LSO neurons in a manner that is directly proportional to the input activity. This inhibition could maintain neurons within their optimum output range for coincidence detection and increase their response bandwidth (Cook et al., 2003).

Reed & Blum (1990) proposed a different function for inhibitory innervation in coincidence detection, specifically in the LSO. Their formulation involved modelling both the excitatory and inhibitory input to the LSO, from the MNTB and antero-ventral cochlear nucleus (aVCN), as being graded based on activation threshold. That is, the neurons of each nucleus (aVCN and MNTB) are arranged such that there is a gradation of low- to high-threshold neurons which are preferentially activated based on the intensity of sound at a given ear. The low-threshold neurons of the inhibitory MNTB innervate LSO neurons that receive high-threshold input from the excitatory AVCN and vice-versa.

With this arrangement, Reed & Blum were able to demonstrate that different ILDs could activate isofrequency columns of LSO neurons in a graded manner, thus, producing a representation of sound localization in a given subset of neurons of the LSO. However, this model assumes that all input arrives coincidently at the LSO from both excitatory and inhibitory neurons and thus simplifies the signal processing and information capacity of this processing center (Abbott & Regehr, 2004). In addition, the robust forms of STP observed at the calyx of Held-MNTB synapse during high-frequency activity add an additional level of complexity to the model of Reed & Blum. Forms of activity-dependent STP form dynamic filters which, in concert with the timing of APs, add further processing latitude to integrating synapses like the LSO (Thomson, 2000; Abbott & Regehr, 2004). Our observations, surrounding bidirectional activity-dependent changes to synaptic delay, may suggest that the timing changes accompanying activity-dependent changes in synaptic strength may produce APs which carry novel information to the LSO.

7.4.4 The Role of Short-Term Plasticity in Coincidence Detection.

Given the evidence that coincidence detection relies on the frequency, timing, and strength of high-frequency input signals, the patterns of STP exhibited upstream in these neurons must also be instrumental in this function. STP is thought to act as a signal filter at relays like the calyx of Held-MNTB synapse (Thomson, 2000; Abbott & Regehr, 2004). Synapses which exhibit STF act as high-pass filters (HPF) through which higher-frequency input produces activity-dependent amplification upon output. These HPF synapses tend to be of low \( P_r \) (Abbott & Regehr, 2004). Depressing synapses tend to act as low-pass filters (LPF) in which high-frequency inputs are quickly attenuated through depletion of the RRP or by some other mechanism (see Zucker & Regehr, 2002). These LPF synapses tend to have higher \( P_r \). Synapses that have intermediate \( P_r \), may exhibit a combination of STF and STD (Figure 6.1) which instils upon them a band-pass like filtering (BPF) property (Abbott & Regehr, 2004). In each case, a
synapses filtering properties are dynamic based on the frequency of input and thus serve a pre-processing function prior to their input reaching an integrator (i.e. MSO/LSO).

Interestingly, of these types of STP (STD, STP, combination of both), STD is found widespread throughout the sensory system of mammals, suggesting that LPFs have a conserved and fundamental role in the processing of sensory stimuli (Fortune & Rose, 2001).

Moreover, the patterns of STP carry information concerning the preceding activity patterns of the neuron. For example, in a depressing synapse a synaptic response, occurring within a short latency of the previous event, will be attenuated as compared to those preceded by longer delays. Thus, having information about the expected magnitude of a response and the kinetics of recovery from STD, a single synaptic event \( \frac{EPSP_{n-1}}{EPSC_n} \) can hold information regarding the incidence of the previous event. This ability is limited by the fact that depressing synapses will eventually reach a steady-state in their responses which does not depend inversely on the input frequency \((1/f\text{ law})\). At this level of STD no information regarding preceding events can be extruded from these later events (Tsodyks & Markram, 1997). For most depressing synapses the “limiting frequency” at which the \(1/f\text{ law} \) no longer applies is quite low (<5Hz) which implies that information concerning preceding events is carried only during the transitionary period between a “rested” and fully-depressed signal, taking approximately four events at high frequencies (Furhmann et al., 2002).

In contrast, facilitating synapses do not have a limiting frequency in the same sense as depressing synapses but rather have a peak frequency at which synaptic reliability and efficacy is maximal and the largest postsynaptic response is produced (Furhmann et al., 2002). The maximal information is carried at an optimal frequency which is proportional to the peak frequency and is higher than that of depressing synapses (Furhmann et al., 2002). Due to this property, a single postsynaptic event carries information regarding at least the preceding eight events. Information concerning events further in the past is only degraded due to the stochastic variability inherent in the release process and the probabilistic uncertainty it establishes within the system (Furhmann et al., 2002).

Given the above, it is intriguing to postulate why sensory systems would favour depressing synapses (Fortune & Rose, 2001). One possibility is that, due to a limited number of AZs, low \( P_r \) synapses may not provide the synaptic reliability required of sensory systems. As above, stochastic variance in quantal release produces uncertainty that reduces the information carrying capacity of a synaptic signal (Furhmann et al., 2002). Another view is that depressing synapses are designed to convey novelty (Thompson, 2000). Since, only the first few events in a train produce a large postsynaptic response, potentially triggering an AP, the perceptive importance of drawn-out stimuli may be attenuated through STD. Moreover, STD may allow for the compression of a wide variety of input signals into a physiologically reasonable pattern of output from the coincident detector, producing a more regular pattern of postsynaptic responses (Goldman et al., 2002; Kuba et al., 2002; Cook et al., 2003; Konishi, 2003). Indeed, STD has been shown to minimize the contribution of strong/fast synapses (high \( P_r \)) allowing for the enhanced detection of weaker/slower ones (Abbott et al., 1997; O'Donovan & Rinzel, 1997). This form of gain control can significantly expand the range of input a single integrating synapse is able to respond to.
Besides altering the pattern of input to the coincident detector, STD has been implicated in advancing the phase of peak response amplitudes in some sensory systems by placing the peak responses at the earliest point in bursts of AP activity (Fortune & Rose, 2001). Likewise, facilitating synapses could delay the phase of peak responses. Phase shifts of the input into coincident detecting neurons could be a means by which the activity of a synapse can temporally shift the activation/inhibition of an integrator creating a type of plasticity-based delay line necessary for a Jeffress model of sound localization coding (Fortune & Rose, 2001; Chance et al., 1998).

However, there is also some evidence to suggest that smaller EPSC/EPSP signals may actually convey a processing advantage over larger ones. Small EPSPs must be exactly coincident in order to provide the coincidence detector with suprathreshold drive, whereas minor temporal discrepancies involving larger EPSPs may accomplish a similar end. As a result, the response window of large EPSPs is significantly wider and less discriminatory than the response window of smaller EPSPs (Kuba et al., 2002). These results suggest that depressed signals may carry more information with greater accuracy than when at full strength. Indeed, animals have been show to localize sound more accurately when the stimulus is of longer duration suggesting that the improved acuity may arise due to STD (Kuba et al., 2002).

From the above it is clear that the characteristics of STP convey a range of different filtering properties onto an input signal and can significantly alter the integrated firing pattern of the target neuron, thus altering the representation of sound source location. The pattern of STP can even hold information regarding previous APs and can temporally shift the response of the target neuron. However, much of the rationale as to why STD is so prevalent in the sensory system is based on models in which multiple excitatory inputs converge to drive a coincidence detector.

The calyx of Held-MNTB synapse shows all of the features of a depressing synapse, in vitro, producing robust signals early in trains followed by rapid depression. Moreover, we have shown that this synapse also exhibits the most rapid and jitter-free $I_{EPSC}$ at the onset of a train of stimuli (Chapter 4 & 6). Our difficulty in rationalizing the role of the calyx of Held-MNTB synapses in the ILD discrimination circuit is that its role at the LSO is inhibitory requiring the re-evaluation of the above hypotheses which attempt to explain the advantageous processing/filtering role of STD.

The rate, extent, and recovery from STD depends on a number of factors including; the frequency of the incoming AP trains, the developmental stage of the synapse, preceding activity, $[\text{Ca}^{2+}]_i$, $[\text{Ca}^{2+}]_o$, second messenger pathways (i.e. GABA, mGluRs), $P_n$, Inactivation of VGCCs, retrograde signalling pathways, postsynaptic receptor desensitization/saturation, and potentially other mechanisms (Trussell & Fischbach, 1989; Jones & Westbrook, 1996; von Gersdorff et al., 1997; Forsythe et al., 1998; Wu & Borst, 1999; Weis et al., 1999; Schneggenburger et al., 2002; Scheuss et al., 2002; von Gersdorff & Borst, 2002; Zucker & Regehr, 2002; Wong et al., 2003; Taschenberger et al., 2005).

The data presented herein focuses primarily on modifications to $P_n$, which are not always straight-forward in their interpretation, based solely on synaptic efficacy (i.e. $I_{EPSC}$). In fact, decreasing $P_n$ can result in an increase in the firing rate and accuracy of postsynaptic APs generated in similar depressing neurons (Brenowitz et al., 1998).
While the calyx of Held exhibits robust STD \textit{in vitro}, it is not clear to what extent it exhibits depression \textit{in vivo}. Recent evidence shows that this synapse may be tonically depressed owing to normal spontaneous activity within the system (Hermann \textit{et al.}, 2007). In the study by Hermann \textit{et al.}, it was hypothesized that this tonic depression served two main purposes. First it increased the rate of recovery from complete depression to the levels observed under spontaneous activity. In order to function as a relay within the sound localization circuit, the calyx of Held must recover from depression with similar kinetics to other synapses within the network. Previously, recovery from depression was significantly longer (hundreds of milliseconds to seconds) than the rapid recovery (tens of ms) observed in hair cells, AN, and CN (Hermann \textit{et al.}, 2007; Wang & Kaczmarek, 1998). Tonic depression appears to bring the STD recovery kinetics of the calyx of Held in line with those of the circuit and may be the result of Ca\textsuperscript{2+} build-up within the terminal (Hermann \textit{et al.}, 2007; Wang & Kaczmarek, 1998). Second, tonic depression may be a means through which the extremely strong LPF property of this synapse is reduced, potentially explaining why the calyx of Held-MNTB synapse has such a high safety-factor for postsynaptic AP generation \textit{in vitro}. Along with tonic depression, a loss in the ability to faithfully transmit presynaptic APs into postsynaptic APs was observed, with transmission failures becoming prevalent later in high-frequency trains (Hermann \textit{et al.}, 2007). In addition, depressed synapses were found to have significantly longer delay times than rested ones, in agreement with our findings (Fedchyshyn & Wang, 2007). The above results, challenging the calyx of Held-MNTB synapse as a simple and faithful relay, further complicate interpretation of the role of inhibitory MNTB inputs to the LSO.

In the LSO of cats, inhibitory drive is strongest at the onset of acoustic stimuli and wanes thereafter, in accordance with the reduction in synaptic strength measured at the calyx during prolonged stimulation (Tsuchitani, 1998). The inhibitory presynaptic terminals originating from the MNTB onto the MSO are primarily glycineergic with a small GABAergic component that declines following P6 (Smith \textit{et al.}, 2000). In the MSO of the rat, IPSCs have fast decay kinetics that increase with development (from 8ms at P6 to 4ms at P14) implying that they are able to keep-up with excitatory inputs of 200Hz (Smith \textit{et al.}, 2000). In the LSO of the gerbil, similar fast IPSCs are measured confirming a similar importance in the timing of inhibitory drive as seen for excitatory drive discussed above (Kotak \textit{et al.}, 1998).

\textit{In vivo}, LSO neurons generate a series of output patterns upon binaural stimulation which encodes the comparison of SPL across the ears. Most of these can be classed as fast or slow chopping responses in which an initial brief burst of activity is followed by a brief silent period (“chopped”) following which activity is resumed in a more regular pattern (Tsuchitani & Johnson, 1985; Tsuchitani, 1998). It is in modifying both the transient early response and the subsequent prolonged response that the depressing nature of the inhibitory input from the MNTB to the LSO most likely contributes to signal processing (Fig. 7.1). In each case, the arrival latency of both the ipsilateral and contralateral input to the LSO is believed to be determined largely by the properties of the aVCN in which the onset of spike firing is dependent on the SPL (Rhode & Smith, 1986). Because of this property, lower SPL signals will reach the LSO with a delay. This is particularly relevant for modulating the early transient bursting
Figure 7.1: Schematic Representation of Excitatory & Inhibitory Integration at the LSO.

(A-C) Schematic representation of excitatory input from the aVCN (green, top panels), inhibitory input from the MNTB (red, top panels), and the integrated output from the LSO (blue, bottom panels) for input arriving coincidentally at the LSO. Examples are illustrated showing equal strength aVCN/MNTB inputs (A), stronger aVCN input (B), and stronger MNTB input (C). (D-F) As in A-C with asynchronous onset of input from the aVCN and MNTB. Examples are illustrated showing stronger and earlier onset MNTB input (D), stronger and later onset MNTB input (E), and weaker and later onset MNTB input (F). For all examples, outer envelope represents the rate of STD and inner frequency envelope represents frequency of APs.
behaviour of the LSO (Tsuchitani, 1998). When the contralateral (inhibitory) signal is strong, the inhibition generated by the MNTB neuron is sufficient to completely shunt excitatory drive from the ipsilateral AVCN (Tsuchitani, 1998). When the contralateral signal is weaker, inhibition arrives at a delay and allows for a brief period of uninhibited excitation to create the short transient burst from the LSO (Fig. 7.1E,F). This burst is “chopped”, to a degree dependent on the strength of the incoming inhibition, as the large amplitude APs from the MNTB arrive at the LSO. Output gradually recovers as depression occurs at the calyx of Held-MNTB synapse. The arrival time of the peak inhibitory drive from the MNTB then determines the length, and even occurrence, of this early signal (Tsuchitani & Johnson, 1985; Tsuchitani, 1998).

Interestingly, following this transient burst, output from the LSO neurons can be quite variable. It appears, based on the work of Tsuchitani (1998), that the depressed output from the MNTB reduces the steady-state discharge rate (gain control) and modulates the subsequent temporal pattern of firing from the LSO. Given this, depressing signals from the MNTB may allow for the immediate localization of sound, based on the timing and occurrence of the fast transient LSO response, followed by the establishment of a delayed steady-state “beating” signal (see Tsuchitani, 1998; Fig. 7 & 8, Fig. 7.1). This “beating” phenomenon may allow for sound to be tracked in the azimuth once detected. If we assume that in the above experiments, performed in anesthetised cats, all spontaneously occurring activity is maintained within the sound localization circuits (see Hermann et al., 2007), then it would appear that even tonically depressed signals from the MNTB are sufficient to completely shunt excitatory drive when timed coincidently.

With the fast kinetics and precise timing of inhibitory drive (Smith et al., 2000), and the output of the LSO being highly modulated by the MNTB, STD, and the factors defining its nature, may be critical in determining the output pattern from the LSO. The bimodal nature of output from the LSO (as above) may serve multiple functions which depend on the magnitude, depression kinetics, timing, and steady-state of inhibition originating at the MNTB.

### 7.4.5 Effects of Release Modality Switching on Signal Processing in the LSO.

In considering how changes in the coupling of VGCCs and SVs alter signal processing in the LSO, we must consider the impact of this form of subsynaptic developmental adaptation on the patterns of STP. Tightening of VGCC-SV coupling, in essence, alters $P_r$ which has a well documented effect on type of STP observed (Zucker & Regehr, 2002). In addition we have shown that, different release modalities have impacts on the timing of SV release and thus the timing of subsequent APs. Indeed, SD increases by hundreds of $\mu$s during STD and SD decreases by hundreds of $\mu$s with development. Although these timing changes may appear insignificant, in a system which must discriminate temporal difference on the order of tens of $\mu$s, changes this large may be considerable (Fedchyshyn & Wang, 2007, Chapter 5).

From our results, alterations in $P_r$, brought about by changes in VGCC-SV coupling, mainly changes the “shape” of the STP observed given a particular input (see Chapter 6). In addition, SD is inversely proportional to $I_{EPSC}$ in immature synapses only, however it is unknown whether $P_r$ decreases...
sufficiently in vivo for the expression of either STF or bidirectionality in SD. The lower \( P \) of immature synapses provides the largest potential for input-output variability, as evidenced by the high measured \( m \) and \( s \) values, and observed in their patterns of STP (Fedchyshyn & Wang, 2005; Chapter 5 & 6). This potential allows greater variability and information carrying capacity based on smaller input variations (Abbott & Regehr, 2004). The advantage of this, if any, in signal processing is unknown but the synapses rapid conversion from micro- to nanodomain release modality upon the onset of hearing suggests that the microdomain modality poses a disadvantage for the accurate processing of sound localization. Alternatively, this plastic stage in development may allow various synapses within the tonotopically arranged nuclei to be “tuned” to a particular set of inputs or prepare for a wide variety of potential input bandwidths (see section 7.3.4). In either case, STD, and the greater reliability offered by the nanodomain modality, appears to allow for the generation of appropriate signals from the MNTB to the LSO.

The rapid depression observed in the mature calyx of Held may allow for precise and sharp chopping of early excitation onset establishing a strong cue for higher processing. Likewise, the rapid depression and sustained steady state transmitted from the mature MNTB may quickly remove complete inhibition from the LSO allowing for the rapid onset of the “beating” behaviour, the processing value of which is also yet unknown. The increased STF observed in immature synapses would then serve to delay the period of maximal inhibition to the LSO, broadening or dulling the transient response and delaying the onset of “beating”. In addition, their lower steady-state responses once depressed may be exhibit insufficient capacity to code for all potential sound frequencies and SPL levels due to insufficient gain control in the LSO (Cook et al., 2003).

SECTION 7.5: CONCLUDING REMARKS

The above suggests that, while a small factor in the overall sound localization circuit, the coupling between VGCCS and SVs provides significant modification to the signals encoding sound placement information. At a low-level, changes in VGCC-SV coupling has the effect of changing the reliance of SV release from a cooperation of VGCCs to the likelihood that a single channel will be activated by an AP. In the middle, at the whole synapse level, release modality tightening provides the basis by which the mature synapse abandons using large \( I_{Ca} \) to generate sufficient SV release, in exchange for using a larger number of AZs, and smaller per-site \( I_{Ca} \) to maintain the same release.

Through this thesis, we have expanded our understanding of VGCC-SV coupling to include its impact on the kinetics of release, the patterns of STP, and have identified a strong candidate for its determination in Septin 5. We have employed some of the most instructive techniques available in electrophysiology to accomplish the above and have used one of the only model systems in the mammalian CNS which provides us the access to do so. In combination with TEM and confocal imaging, we have provided strong evidence for our conclusions and have hinted at numerous avenues of potential future study.
Despite the evidence presented herein, we have kept a somewhat one-sided view of the synapse in that we have largely tested and considered only presynaptic explanations for our findings. We have assumed that the postsynaptic MNTB neuron is a faithful reporter of our presynaptic manipulations, which it may be more so in some experimental circumstances than in others. However, we have tried to address these possibilities throughout this dissertation in rationale if not by experimentation.

As above, and at the highest level, we have attempted to anticipate the impact of changes to VGCC-SV coupling on the nature and processing of the inhibitory signal to the LSO. However, these postulations are primarily extrapolations based on the current data that appear to fit within the framework of existing systems literature. Using an in vitro slice preparation allows for the experimental manipulations presented herein, however, its controllable nature comes at the expense of sound higher-level conclusions. Many inhibitory and excitatory connections are either severed or blocked in our preparation making direct comparison of this work with in vivo findings difficult. Extension of this preparation to maintain connectivity throughout the network, and furthermore be able to find connected sets of neurons, would prove extremely difficult experimentally.

Perhaps the most we may be able to reasonably extract, using the techniques presented here, is an in depth understanding of each relay in the network. Using stimuli based on off-line in vivo recordings may provide an image of a neurons steady-state if one exists at all (Hermann et al., 2007). Perhaps with modification to our preparation, two-synapse relays could be held intact for future study.

Finally, this dissertation has relied completely on $I_{EPSC}$ as a readout of presynaptic activity. While this is obviously the best measure for the purpose of evaluating quantal release, APs are the currency of information transfer in the CNS (Sanes, 1990; Thompson, 2003). In extending these finding to network behaviour, we must understand how changes observed in $I_{EPSC}$ alter the amplitude and timing of AP generation in the MNTB in order to competently assay their integrative role at the LSO. Simple postsynaptic current-clamp recordings, performed in experiments similar to those in Chapter 6, would lend more direct insight into the implications of release modality tightening on processing in the LSO.

Apart from the fundamental limitations outlined above, our results provide evidence of a novel form of subsynaptic plasticity, while also characterizing its implications on STP and determining its apparent underlying substrate. At the level of the synapse, the intended target of this work, we have built significantly upon the understanding of the presynaptic release apparatus and demonstrated that it cannot be considered a static entity, either physically or functionally.

Synaptic development results in an elegantly coordinated series of changes, both obvious and subtle, that ultimately converge to consolidate the fundamental processes of synaptic transmission, signal processing, and ultimately perception. The conversion between microdomain and nanodomain release modalities appears to be one important milestone in this dynamic and complex process.
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9. APPENDICES
9.1 THE EFFECTS OF BACLOFEN ON ACTIVITY-DEPENDENT CHANGES IN TEMPORAL DELAYS

To address whether the progression in delay and the increase in variance could be affected by presynaptic neuromodulation such as activation of metabotropic receptors, we repeated experiments in the presence of 50 μM of the GABABR agonist baclofen in 2mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 9.1.1). Baclofen acts through a G-protein mediated signalling cascade (G\textsubscript{βγ}) to slow the activation of P/Q-type Ca\textsuperscript{2+} channels (Isaacson, 1998; Takahashi et al., 1998; Kajikawa et al., 2001), the type predominantly mediating release of neurotransmitter in the calyx of Held at this developmental stage (Forsythe et al., 1998; Iwasaki et al., 2000). Upon delivery of high frequency axon-evoked stimulation, in the presence of baclofen (2mM [Ca\textsuperscript{2+}]\textsubscript{o}), S was significantly decreased for both delay and variance with pooled progression rates shown in Table 4.2A & B respectively. The extent to which these parameters increased was also reduced in the presence of baclofen with pooled values shown in Table 4.2C & D respectively. In line with other experiments conducted in reduced [Ca\textsuperscript{2+}]\textsubscript{o} or increased Ca\textsuperscript{2+} buffer (EGTA-AM), baclofen attenuates activity-dependent increases in temporal delays and variance by reducing Ca\textsuperscript{2+} influx per AP and hence reducing the accumulation of residual Ca\textsuperscript{2+} during repetitive activity. The above results imply that physiological activation of GABAB receptors could be a manner through which tonic inhibitory activity may aid in maintaining the fidelity of transmission during repetitive activity.
Figure 9.1.1: The Effects of Baclofen on Activity-Dependent Changes in Temporal Delays. (A) Examples of first (black lines) and last (grey lines) pairs of presynaptic currents and postsynaptic EPSCs evoked by thirty sweeps of train stimuli (200Hz, 150ms) in 2mM [Ca$^{2+}$], w/50μM Baclofen. (B) Envelope plots for delay components against the number of stimuli in a train under the experimental conditions described in A. Solid black lines represent linear regression to scattered data points shown. Dashed red lines represent linear regression to scattered data shown in Figure 4.4C.
9.2 REPLENISHMENT OF THE RRP IN Sept5⁻/⁻ & Sept5⁺/+ SYNAPSES

9.2.1 Sept5 Is Not Crucial For the Replenishment of the Readily Releasable Pool of Synaptic Vesicles.

Having provided evidence implicating Sept5, or Sept5 containing filaments, in determining VGCC-SV coupling in the immature calyx of Held, we next asked whether, Sept5 may also bind to syntaxin molecules between SVs, tethering them together. Filaments, of unknown composition, have been shown to exist between SVs near the AZ (Hirokawa et al., 1989). In light of these observations, we asked whether these filaments may contain or be comprised of Sept5 and may serve a role in the transiting of SVs to the AZ from the readily releasable pool of SVs (RRP) following depletion.

The pool of synaptic vesicles can be subdivided into two main components; those that are releasable under conditions of typical activity (RRP) and those that refill the RRP, or the reserve pool (RVP) (reviewed in Neher, 1998). We further subdivided the RRP into an immediately releasable pool (IM-RRP) and an intermediate pool (IT-RRP) of synaptic vesicles which are separable through the careful control of $I_{Ca}$, which can differentially recruit SVs from each. The IM-RRP is defined as the pool consisting of those SVs that are available for release given that all VGCCs flux a tail current-like $I_{Ca}$. These SVs are most likely those in close proximity to the VGCCs and contain the subset of SVs released, in a probabilistic manner, in response to single AP events. In contrast, the IT-RRP consists of SVs which are recruited and released during sustained activity or sustained elevations in intra-terminal $[Ca^{2+}]$ and include those that we have defined as the IM-RRP. The IT-RRP, as defined above, is analogous to the classical definition of the RRP determined from the calyx of Held (Schneggenburger et al., 1999; Taschenberger & von Gersdorff, 2000; Meyer et al., 2001; Sakaba & Neher, 2001). We hypothesized that, if Sept5 was an integral component of filaments tethering SVs together and aided in their transiting to the AZ, then loss of Sept5 should result in a RRP replenishment deficiency in Sept5⁻/⁻ synapses. In addition, by separating the IM-RRP and IT-RRP components of the RRP, we can further isolate the role of Sept5, if any, in this process.

To determine whether loss of Sept5 would reduce the effectiveness of IM-RRP replenishment, we depolarized voltage-clamped presynaptic terminals from -80mV to 40mV for 4ms. This protocol pushes VGCCs past their effective reversal potential for $Ca^{2+}$ and to a potential at which maximum open probability occurs (Augustine et al., 1985; Llinás et al., 1989; Borst & Sakmann, 1998) (Fig. 9.2.1A). Upon repolarization, a large $Ca^{2+}$ tail-current is generated in the short period before VGCC closing. In response, $Ca^{2+}$ microdomains are formed which are analogous to those generated in response to APs, however, domains are formed at all VGCCs rather than the small subset normally activated during an AP (Augustine et al., 1987; Borst & Sakmann, 1998; Gentile & Stanley, 2005; Yang & Wang, 2005). The resulting synaptic output ($I_{EPSC}$) contains those SVs available to a single AP and defined as the IM-RRP.

By separating two such depolarizations by a $\Delta t$ of increasing duration (from 1ms to 10s), we determined the rate of recovery of this portion of the RRP in both immature and mature Sept5⁺/+ and Sept5⁻/⁻ synapses (Fig. 9.2.1B,C). In immature synapses, at the shortest $\Delta t$ possible (1ms), percent
Figure 9.2.1: Replenishment of the Immediately Readily Releasable Pool of Synaptic Vesicles in Sept5\textsuperscript{++} & Sept5\textsuperscript{--} Synapses.

(A) Presynaptic voltage-command protocol used to measure the recovery of the immediately readily releasable pool of synaptic vesicles. Two voltage steps (-80mV to +40mV, 4ms), separated by increasing \( \Delta t \) (intervals ranging from 1ms to 10s), are used to evoke \( I_{\text{ca}} \) and \( I_{\text{EPSC}} \) from voltage-clamped calyces and their corresponding MNTB neurons. (B) Paired presynaptic \( I_{\text{ca}} \) and postsynaptic \( I_{\text{EPSC}} \) recordings, in response to the voltage-command protocol shown in A, for immature Sept5\textsuperscript{++} (top panel) and Sept5\textsuperscript{--} (bottom panel) synapses. \( \Delta t \) ranges from 1ms to 100ms are shown. Magnification of traces at selected intervals (\( \Delta t=1\text{ms}, 100\text{ms}, 1\text{s}, 4\text{s}, \text{and } 10\text{s} \)) are shown superimposed at right. (C) As in B for mature Sept5\textsuperscript{++} (top panel) and Sept5\textsuperscript{--} (bottom panel) synapses in response to the voltage-command protocol shown in A. (D) Summary Data showing the recovery of the immediately readily releasable pool of synaptic vesicles against the range of \( \Delta t \) tested for immature Sept5\textsuperscript{++} and Sept5\textsuperscript{--} synapses. (E) As in D for mature Sept5\textsuperscript{++} and Sept5\textsuperscript{--} synapses.
recovery was not significantly different between Sept5+/+ and Sept5−/− (n=9/6) synapses being 38±10 and 28±4% respectively (Fig. 9.2.1B). Overlaying I_{Ca} and I_{EPSC} from the initial step (black) and Δt of 1ms (red), 100ms (blue), 1s (green), 4s (cyan), and 10s (magenta) (Fig. 9.2.1B, right panel), the relative recovery of the I_{EPSC} and the stability of the I_{Ca} can be seen for both immature Sept5+/+ and Sept5−/− synapses. Recovery was well described by a bi-phasic exponential with time constants of 90±12 and 5000±800ms vs. 67±7 and 6000±2000ms for Sept5+/+ and Sept5−/− synapses respectively (Fig. 9.2.1D). None of the time constants or individual points, at various Δt on the recovery curves, could be considered significantly different (Student’s t-test, p>0.05).

In mature synapses, even the shortest Δt (1ms) could not significantly deplete the IM-RRP with initial recovery of 53±4 and 49±9% (n=10/6) for Sept5+/+ and Sept5−/− synapses respectively (Fig. 9.2.1C). Neither fast (50±15 vs. 70±17ms) or slow (6000±1000 vs. 6000±1200ms) time constants were found to be significantly different between mature Sept5+/+ and Sept5−/− synapses (Fig. 9.2.1E), suggesting that Sept5 is not essential for transiting SVs to the AZ and into the IM-RRP of SVs.

While Sept5 may not be crucial for trafficking SVs to the IM-RRP, it may be involved in refilling the broader IT-RRP which depletes during sustained activity (Weis et. al., 1999). To investigate whether Sept5 is required for the replenishment of the IT-RRP, we depolarized voltage-clamped presynaptic calyces from −80mV to 0mV for 10ms (Fig. 9.2.2A). During these longer voltage steps, a large sustained I_{Ca} was recorded while MNTB neurons registered a large sustained I_{EPSC} that decayed to near-baseline suggesting that the RRP had been largely depleted (Fig. 9.2.2B,C). Voltage steps of this type are thought to trigger release of a pool of SVs larger than those depleted during high-frequency activity at the calyx of Held, which we have defined above as the IT-RRP (reviewed in Schneggenburger et al., 2002).

To assay the effect of a loss of Sept5 on recovery of the IT-RRP, we separated two depolarizing steps by an increasing Δt (10ms to 10s) and measured the recovery of I_{EPSC} in immature and mature Sept5+/+ and Sept5−/− synapses. This protocol effectively depleted a majority of the IT-RRP in immature synapses at the shortest Δt (10ms), with recovery of 2.5±0.5 and 7±2% (n=12/8) observed for Sept5+/+ and Sept5−/− synapses respectively (Fig. 9.2.2B).

Mature synapses depleted to a lesser extent with percent recovery being 10±1 and 17±3% (Δt=10ms, n=8/6) for Sept5+/+ and Sept5−/− synapses respectively (Fig. 9.2.2C). Recovery of the IT-RRP was also well described with a bi-phasic exponential with fast time constants of 170±11 and 160±17ms for immature and 130±10 and 150±12ms for mature Sept5+/+ and Sept5−/− synapses respectively (Fig. 9.2.2D,E). Slow time constants were 9000±1000 and 6000±800ms for immature and 10000±2000 and 15000±3000ms for mature Sept5+/+ and Sept5−/− synapses respectively. These time constants are in line with estimates from previous reports at this and other central synapses for recovery of the RRP (Stevens & Tsujimoto, 1995; Dobrunz & Stevens, 1997; Wang & Kaczmarek, 1998; Weis et. al., 1999). Despite sizable differences in the time constants, none of the individual data points or time constants for recovery of the IT-RRP proved to be statistically significant between Sept5+/+ and Sept5−/− synapses, in either the immature or mature experimental groups (Student’s t-test, p>0.05).
Figure 9.2.2: Replenishment of the Readily Releasable Pool of Synaptic Vesicles in \textit{Sept5}^{+/+} & \textit{Sept5}^{-/-} Synapses.

(A) Presynaptic voltage-command protocol used to measure the recovery of the readily releasable pool of synaptic vesicles. Two voltage steps (-80 to 0mV, 10ms), separated by increasing \( \Delta t \) (intervals ranging from 10ms to 15s), are used to evoke \( I_{Ca} \) and \( I_{EPSC} \) from voltage-clamped presynaptic calyces and their corresponding postsynaptic MNTB neurons. (B) Paired presynaptic \( I_{Ca} \) and postsynaptic \( I_{EPSC} \) recordings, in response to the voltage-command protocol shown in A, for immature \textit{Sept5}^{+/+} (top panel) and \textit{Sept5}^{-/-} (bottom panel) synapses. \( \Delta t \) ranges from 20ms to 1s are shown. Magnification of traces at selected intervals (\( \Delta t=\)Initial, 10ms, 500ms, 1s, 4s, 10s) are shown superimposed at right. (C) As in B for mature \textit{Sept5}^{+/+} (top panel) and \textit{Sept5}^{-/-} (bottom panel) synapses in response to the voltage-command shown in A. (D) Summary data showing the recovery of the readily releasable pool of synaptic vesicles against the range of \( \Delta t \) values tested (10ms to 15s) for immature \textit{Sept5}^{+/+} and \textit{Sept5}^{-/-} synapses. (E) As in D for mature \textit{Sept5}^{+/+} and \textit{Sept5}^{-/-} synapses.
as observed for the IM-RRP, replenishment of the IT-RRP does not seem to require Sept5, suggesting that, while the filamentous structures found between SVs may contain various members of the septin family of proteins, Sept5 is either absent or functionally redundant in serving this role at both the immature and mature calyx of Held nerve terminal.

9.2.2 Trains of Evoked Stimulation Uncover Elevated Release Probability in Immature Sept5−/− Synapses.

Having determined that loss of Sept5 had no significant effect on replenishment of the RRP, we sought to confirm this observation using an experimental approach that did not disturb the intracellular environment of the calyx. We used trains of axon-evoked APs, in 2mM [Ca2+]o, to generate depressing trains of IEPSC in immature and mature Sept5+/+ and Sept5−/− synapses. In immature synapses, stimulation trains were 200ms in duration at 100Hz while in mature synapses they were 100ms in duration at 200Hz. Both trains sufficiently depleted the RRP, at each developmental stage, as IEPSC reached plateau amplitudes nearing the end of the trains, indicative of a balance between release from the RRP and refilling from the RVP (Wang & Kaczmarek, 1998; Schneggenberger et al., 1999). To assay replenishment of the RRP under these conditions, two such trains were separated by a Δt of increasing duration (50ms to 15s) from which the percent recovery of the RRP was calculated (Fig. 9.2.3A). In all experiments the first tEPSC peak, cumulative tEPSC peak amplitude, and the cumulative tEPSC current density was taken as a measure of the transmitter released during the train. All three measures showed identical trends, therefore only cumulative tEPSC density was chosen to illustrate recovery of the RRP.

When we performed these experiments in immature Sept5+/+ and Sept5−/− synapses we found that Sept5−/− synapses showed a decreased replenishment at the shortest Δt (50ms) as compared with Sept5+/+ synapses (53±2 vs. 63±3%, p<0.05) (Fig. 9.2.3D). When the time constants of recovery were analyzed we found that there was no significant difference between fast or slow components in Sept5+/+ and Sept5−/− synapses (200±40 vs. 200±40ms; 3200±300 vs. 3200±300ms, p>0.05) which translated into the Sept5−/− synapses having a consistently lower percent recovery for all time points between 50ms and 2s (Fig. 9.2.3B,D). So while the Sept5−/− synapses show a deficit in initial recovery, thereafter, both Sept5+/+ and Sept5−/− synapses recovered from depletion of the RRP at the same rates. In contrast, mature Sept5+/+ and Sept5−/− synapses showed no difference in their recovery kinetics or in the extent to which they recovered initially (54±1 vs. 51±2% at 50ms; 520±150 vs. 520±100ms; 3000±500 vs. 4000±600ms, p>0.05) (Fig. 9.2.3C,E). The initial deficit in RRP recovery observed in immature Sept5−/− synapses could be explained if an elevated P, caused more SV release in Sept5−/− than in Sept5+/+ synapses. If this were true, and the recovery rate of the RRP were similar, it would be reflected in the downward shifted normalized recovery curve observed in immature Sept5−/− synapses. As discussed in Chapter 5, both immature and mature Sept5−/− synapses show increased RRP size as compared to Sept5+/+ synapses. When we compared the cumulative current density, measured from the initial depleting train of tEPSC, between immature Sept5+/+ and Sept5−/− synapses, we found that the later was releasing significantly more transmitter during the train than the former (43000±3000 vs.
Figure 9.2.3: Recovery of the Readily Releasable Pool in Response to High-Frequency Stimulation. (A) Afferent stimulation protocol. Two trains of stimuli, 100Hz for 200ms in immature and 200Hz for 100ms in mature synapses, are separated by Δt ranging from 150ms to 15s. (B) Postsynaptic $I_{\text{EPSC}}$ in response to the stimulation protocol shown in A, for Δt of 50ms, 1s, and 10s in immature Sept5$^{\text{+}}$ (top panel) and Sept5$^{-}$ (bottom panel) synapses. (C) As in B for mature Sept5$^{\text{+}}$ (top panel) and Sept5$^{-}$ (bottom panel) synapses. (D) Summary data plotting the recovery of the cumulative area of EPSC trains as a function of Δt in immature Sept5$^{\text{+}}$ and Sept5$^{-}$ synapses. Solid lines are biphasic exponential curves fitted to the data shown. (E) As in D for mature Sept5$^{\text{+}}$ and Sept5$^{-}$ synapses. (F) Summary data showing cumulative EPSC area for the initial and second (Δt=100ms) EPSC trains for both immature Sept5$^{\text{+}}$ and Sept5$^{-}$ synapses. Asterisk denotes significance to p<0.05 (Student's t-test). (G) Summary plot of normalized $I_{\text{EPSC}}$, from the initial EPSC train, against event number for immature Sept5$^{\text{+}}$ (closed circles) and Sept5$^{-}$ (open diamonds) synapses. Solid lines are monophasic exponential curves fitted to the data shown.
35000±2000pA•sec⁻¹•events, Student’s t-test, p<0.05). 50ms following the initial depleting train, both synapses had statistically similar cumulative current densities (24000±3000 vs. 22000±2000pA•sec⁻¹•event) suggesting that they had recovered the same number of SVs in that time but the Sept5⁺ synapses had expended more SVs initially due to their higher Pr (Fig. 9.2.3F). Thus, given the same rates of recovery for immature Sept5⁺/− and Sept5⁻/− synapses, the apparent deficit in Sept5⁺/− recovery would persist until the curves converged on their asymptote (100%) at longer Δt (larger than 2s) (Fig. 9.2.3D). Interestingly, a difference in the initial I_{EPSC} density was observed in mature Sept5⁺/− and Sept5⁻/− synapses also (67±6 vs. 43±4nA•ms, n=6/8 respectively, p<0.05), which also persisted into the second train of I_{EPSC} (35±5 vs. 23±3nA•ms, Sept5⁻/− vs. Sept5⁺/−, P<0.05), resulting in the identical replenishment curves observed (Fig. 9.2.3E).

As an additional measure of elevated Pr, we plotted the normalized I_{EPSC}, as a function of event number, to determine whether Sept5⁺/− synapses depressed more rapidly, and to a greater extent, than Sept5⁻/− synapses. Fitting the normalized depression pattern with a single exponential revealed that immature Sept5⁺ synapses had a significantly faster depression rate than Sept5⁺/− synapses (1.9±0.7 vs. 2.3±0.1events, Student’s t-test, p<0.05) (Fig. 9.2.3G). The extent of depression was also significantly greater in immature Sept5⁺ synapses than in Sept5⁺/− synapses (17.8±0.6 vs. 15±0.5%, Student’s t-test, p<0.05) confirming that immature Sept5⁺/− synapses do have a slightly elevated Pr. An elevated Pr, in line with the observation that SVs in immature Sept5⁺ synapses are located in closer proximity to the AZs which would bring them closer to VGCCs, than in Sept5⁻/− synapses, increasing their Pr. Mature synapses showed the opposite characteristics in STD with Sept5⁺ synapses depressing less rapidly and to a lesser extent than Sept5⁺/− synapses (data not shown). This is likely due to the increased RRP size observed in these synapses which is in agreement with the larger number of morphologically docked SVs observed in these synapses (Fig. 9.2.5D).

### 9.2.3 Sept5 Aids in Defining the Size of the Readily Releasable Pool of Synaptic Vesicles Under Conditions of Large I_{Ca} In Immature & Mature Synapses.

As a final assay to determine whether Sept5 is involved in the replenishment of the RRP, we repeated the experiments shown in Fig. 9.2.3 but in the presence of 0.2mM TEA. This concentration of TEA effectively prolongs the width of presynaptic APs by partially blocking K⁺ channels, resulting in increased Ca²⁺ influx and SV release. Increasing intra-terminal [Ca²⁺], either through increasing activity or broadening APs, has been shown to increase the rate of recovery of the RRP at the juvenile calyx of Held synapse (Wang & Kaczmarek, 1998). We hypothesized that the loss of Sept5 may not only result in SVs residing more closely to the AZ, but may be involved in the Ca²⁺-dependent increase in RRP replenishment kinetics observed by Wang & Kaczmarek (1998) when synapses are challenged with significantly high Ca²⁺ signals.

In immature Sept5⁺/− and Sept5⁻/− synapses, we separated two depleting afferent-evoked stimulus trains (100Hz for 200ms, 2mM [Ca²⁺]₀) by an increasing Δt (50ms to 15s) and analyzed the recovery of the cumulative current density (Fig. 9.2.4A). Due to the large I_{EPSC} generated in the presence of TEA, we
Figure 9.2.4: Ca\textsuperscript{2+}-Dependent Recruitment of the Readily Releasable Pool of Synaptic Vesicles.

(A) Postsynaptic EPSC\textsubscript{epsc} in response to the stimulation protocol shown in Figure 5.11A, for $\Delta t$ of 50ms, 1s, and 10s for Immature Sept\textsuperscript{5+/-} (top panel) and Sept\textsuperscript{5-} (bottom panel) synapses in the presence of 50 mM CTZ, 1mM KYN, and 0.2 mM TEA. (B) As in A for mature Sept\textsuperscript{5+/-} (top panel) and Sept\textsuperscript{5-} (bottom panel) synapses in the presence of 50 mM CTZ and 0.2 mM TEA. (C) Summary data plotting the recovery of the cumulative area of EPSC trains as a function of $\Delta t$ duration in immature Sept\textsuperscript{5+/-} and Sept\textsuperscript{5-} synapses. Solid lines are biphasic exponential curves fitted to the data shown. (D) As in C for mature Sept\textsuperscript{5+/-} and Sept\textsuperscript{5-} synapses. (E) Summary data showing cumulative EPSC area for the initial and second ($\Delta t=100$ms) EPSC trains for both mature Sept\textsuperscript{5+/-} and Sept\textsuperscript{5-} synapses in the presence of TEA and CTZ. Asterisk denotes significance to $p<0.05$ (Student’s t-test). (F) Summary plot of normalized EPSC amplitude, from the initial EPSC train, against event number for mature Sept\textsuperscript{5+/-} (closed circles) and Sept\textsuperscript{5-} (open diamonds) synapses in the presence of TEA and CTZ. Solid lines are biphasic exponential curves fitted to the data shown.
added 50µM CTZ to block AMPAR desensitization and 1mM KYN to prevent AMPAR saturation (Neher & Sakaba, 2001). Addition of KYN reduced the amplitude of $I_{EPSC}$ to levels similar to those in the absence of TEA while addition of CTZ significantly broadened $I_{EPSC}$ (Fig. 9.2.4B). When we plotted the percent recovery of the normalized $I_{EPSC}$ density against $\Delta t$ we found no significant difference in the curves between immature $\text{Sept}5^{\text{+/+}}$ and $\text{Sept}5^{-/-}$ synapses (Fig. 9.2.4D). In fact, addition of TEA, CTZ, and KYN decreased the rates of RRP replenishment relative to both $\text{Sept}5^{\text{+/+}}$ and $\text{Sept}5^{-/-}$ immature synapses in the absence of these pharmacological agents, in contrast to similar experiments performed later in development (Wang & Kaczmarek, 1998). Fast and slow time constants, in the presence of TEA, CTZ, and KYN were 290±40 and 5100±300ms (n=7) for $\text{Sept}5^{\text{+/+}}$ synapses and 320±50 and 5700±400ms (n=11) for $\text{Sept}5^{-/-}$ synapses (Fig. 9.2.4D). The enhancement in RRP current density was maintained with the additions of TEA, KYN, and CTZ with immature $\text{Sept}5^{-/-}$ synapses having values of 58.1±0.4nA•ms and $\text{Sept}5^{+/+}$ synapses having values of 45±1nA•ms (Fig. 9.2.5A). Initial train $I_{EPSC}$ densities were also significantly larger in $\text{Sept}5^{-/-}$ synapses 89±9nA•ms vs. 70±6nA•ms in $\text{Sept}5^{+/+}$ synapses.

In mature $\text{Sept}5^{+/+}$ and $\text{Sept}5^{-/-}$ synapses, we performed similar analyses, in the presence of TEA and CTZ, using trains of afferent-evoked APs of 100ms and 200Hz. Postsynaptic $I_{EPSC}$ were broader with TEA and CTZ in comparison to those without and increased in amplitude as expected (Fig. 9.2.4C). When we plotted recovery as a function of $\Delta t$ we found that the time constants of recovery were not statistically different between $\text{Sept}5^{+/+}$ and $\text{Sept}5^{-/-}$ synapses but were faster than those measured in the absence of TEA (190±30 vs. 230±35ms; 3000±600 vs. 4000±500ms; p>0.05, n=7/11) (Fig. 9.2.4C) as expected at this developmental stage (Wang & Kaczmarek, 1998). However, the $\text{Sept}5^{-/-}$ synapses began at a lower initial recovery level than did $\text{Sept}5^{+/+}$ synapses for the shortest $\Delta t$ of 50ms (42±2 vs. 57±2, p<0.05, n=9/10). As in the immature synapses in the absence of TEA (Fig. 9.2.3D), the kinetics of recovery were unaltered by the loss of Sept5. Interestingly, in these synapses, neither the $I_{EPSC}$ density from the first or second train was significantly different (Fig. 9.2.4E). Plotting the normalized STD we found little difference between $\text{Sept}5^{+/+}$ and $\text{Sept}5^{-/-}$ synapses at this developmental stage (Fig. 9.2.4F). These data suggest that the P, is similar between the genotypes in mature synapses under these experimental conditions.

The observed difference in RRP current density, in the absence of TEA, was reduced but still significant between $\text{Sept}5^{+/+}$ and $\text{Sept}5^{-/-}$ synapses with values of 98±4 and 90±1nA•ms respectively (Fig. 9.2.5B).

### 9.2.4 General Discussion on the Role of Sept5 in Defining and Replenishment of the Readily Releasable Pool of synaptic Vesicles.

Given that syntaxin-containing 7S complexes exist on SVs (Otto et al., 1997), it is reasonable to ask whether Sept5 plays a role in transiting SVs to the AZ locations where SVs had been depleted previously. Filaments approximately 30nm long, believed to be synapsin, have been observed linking SVs together and are hypothesized to play a role in the trafficking of SVs, both between pools and to the
Figure 9.2.5: Readily Releasable Pool Size in Sept5+/+ & Sept5−/− Synapses in the Presence of TEA & the Distribution of Synaptic Vesicles.

(A) Cumulative I_{EPSC} density as a function of event number for immature Sept5+/+ (black line) and Sept5−/− (red line) synapses evaluated from the I_{EPSC} trains of experiments shown in Fig. 9.2.4A,C, in the presence of 1mM KYN, 50µM CTZ, and 0.2mM TEA. (B) Cumulative I_{EPSC} density as a function of event number for mature Sept5+/+ (black line) and Sept5−/− (red line) synapses, evaluated from the I_{EPSC} trains of experiments shown in Fig. 9.2.4B,D, in the presence of 50µM CTZ and 0.2mM TEA. Dashed lines represent upper and lower 95% confidence limits for linear regression fit of points in the plateau phase of each cumulative amplitude plot. (C) Normalized cumulative histograms of SV-AZ separation distance measured in immature (P8-12, black line) and mature (P16-18, grey line) synapses. (D) As in C for immature Sept5−/− (dashed red line) and Sept5+/+ (black line) synapses. Distributions in C and D are statistically different (K-S test, P<0.05). Asterisks in A and B indicate significance (P<0.05, Student’s t-test).
AZ (Hirokawa et al., 1989; reviewed in Dousseau & Augustine, 2000). However, insights gained from synapsin I and/or II null mice have been difficult to interpret. Loss of synapsin I has been shown to have only subtle effects on the release probability of SVs and no effect on maintenance of synaptic transmission (Rosahl et al., 1995). Loss of synapsin II, in contrast, caused increased depression during sustained activity and caused a decrease in the number, but not the distribution, of SVs clustered at AZs (Rosahl et al., 1995). The decreased number of SVs made interpretation of the increased depression rate in synapsin I and II synapses difficult as deficits to either the kinetics of exocytosis or the replenishment rate of the RRP could underlie the observations (Sun et al., 2006). Recent studies from immature Calyx of Held-MNTB synapses suggest that loss of synapsin I and II seem to selectively affect the P_r of SVs released during the steady-state of high-frequency trains, but not the refilling rate of the RRP (Sun et al., 2006). This raises the possibility that Sept5, binding to syntaxin proteins between SVs, may function to transit SVs to the RRP after depletion. In addition, this may provide an alternate role for Sept5 in mature synapses.

Using a number of assays to separate distinct pools of SVs (IM-RRP vs. IT-RRP) we found no difference in the rate of replenishment of the RRP between Sept5^{+/+} and Sept5^{-/-} synapses at either developmental stage (Fig. 9.2.1, 9.2.2). Using a non-invasive approach (see Wang & Kaczmarek, 1998) we again assayed the replenishment rates of the RRP in immature and mature synapses and found some interesting results. In immature synapses, the loss of Sept5 appears to increase the size of the RRP and the number of SVs released during a trains of EPSCs (Fig. 9.2.3). Interestingly, the kinetics of and replenishment of the RRP were not different between Sept5^{+/+} and Sept5^{-/-} synapses, however, the number of SVs that were recruited during the initial trains were significantly greater. These results imply that the enhanced P_r, observed in immature Sept5^{-/-}, presumably due to tighter VGCC-SV coupling, results in a greater number of SVs being released during the first train. However, the number of SVs that may be replenished between the first and second trains of EPSCs (i.e. Δt=50ms), are similar between the genotypes. This potentially explains the downward shift in the RRP recovery curves for immature Sept5^{-/-} synapses relative to Sept5^{+/+} synapses.

Mature Sept5^{-/-} synapses, in contrast to immature ones, do not show any difference in the rate or extent of recovery of the RRP but do show enhanced RRP and SV release during initial trains, similar to immature Sept5^{-/-} synapses. This implies that while more SVs are released during the first trains, the mature synapse is able to recover the same proportion of these SVs in Sept5^{-/-} or Sept5^{+/+} synapses. This may be the result of maturation in the endocytotic machinery which is capable of recycling a greater number of SVs in the mature synapse. In the immature synapse, the rate of recycling may be limited such that the increased P_r resulting from the loss of Sept5 depletes SVs more readily than they are replenished (Fig. 9.2.3). With maturity, an increase in the SV recycling rate may have the headroom to recover the increased number of SVs released during the initial trains. Direct capacitive recordings from Sept5^{-/-} and Sept5^{+/+} calyces will provide a more direct measure of the accuracy of this hypothesis.

When immature and mature Sept5^{-/-} and Sept5^{+/+} were challenged with larger I_{Ca}, due to AP broadening with TEA, immature synapses showed no difference in their rate or extent of their RRP recovery. However, immature Sept5^{-/-} synapses release a greater number of SVs during first trains and
had a larger RRP than did Sept5+/− synapses, similar to our previous findings without TEA. These findings are difficult to interpret as we would expect a downward shift in the RRP recovery curves if the rationale proposed above held in the presence of TEA. Also confusing is the observation that the kinetics of RRP refilling are slowed with TEA for both immature Sept5+/− and Sept5−/− synapses. This may be a result of the large number of quanta released during these trains, however, elevations in ICa have been shown to increase the rate of RRP refilling under similar conditions, albeit in slightly older synapses (Wang & Kaczmarek, 1998). It is possible that the increase in ICa is increasing the rate of SV recycling, as in Wang & Kaczmarek (1998), allowing for enough headroom in the process to support the higher P, and SV output of the Sept5−/− synapses, similar to mature synapses without TEA (see above).

Interestingly, mature Sept5−/− synapses recovered their RRP to a lesser degree, but with the same kinetics, as Sept5+/− synapses when challenged with high ICa (Fig. 9.2.4). In this case, the difference in RRP current density was reduced but still slightly larger in Sept5−/− synapses than in Sept5+/− synapses (Fig. 9.2.5). It is possible that a slight increase in the first train IEPSC density and a slight decrease in the second trains IEPSC density, neither statistically significant, may be sufficient to shift the RRP recovery curve downward as observed. It is likely that whatever effect the loss of Sept5 has on defining the size of the RRP, is overwhelmed by the increased ICa signal into mature synapses in the presence of TEA. In this case, the recovery headroom of SVs may again become limiting when larger portions of the RRP are recruited under conditions of increased ICa.

From the data above, it is clear that, while Sept5 does not appear to play a role in defining the RRP under conditions of invasive experimentation (i.e. presynaptic whole-cell recordings), it seems to allow for a larger RRP to be recruited for release in non-invasive experimentation. This finding is true of both immature and mature synapses with both regular and enhanced ICa. Despite this consistency, the mechanisms of action of Sept5 in accomplishing this are unclear based on the above findings. Using presynaptic capacitance recordings, a more direct measure of RRP recovery, would reduce the complexity in evaluating the role of Sept5 from processes potentially monitored a few steps downstream of their actual site of action (i.e. endocytosis).

Given that syntaxin-containing 7S complexes exist on SVs (Otto et al., 1997) it is reasonable to ask whether Sept5 is involved in transiting SVs to the AZ locations where SVs had been depleted previously. Filaments approximately 30nm long, believed to be synapsin, have been observed linking SVs together and are hypothesized to play a role in the trafficking of SVs, both between pools and to the AZ (Hirokawa et al., 1989; reviewed in Dousseau & Augustine, 2000). However, insights gained from synapsin I and/or II null mice have been difficult to interpret. Loss of synapsin I has been shown to have only subtle effects on the release probability of SVs and no effect on maintenance of synaptic transmission (Rosahl et al., 1995). Loss of synapsin II, in contrast, caused increased depression during sustained activity and caused a decrease in the number, but not the distribution, of SVs clustered at AZs (Rosahl et al., 1995). The decreased number of SVs made interpretation of the increased depression rate in synapsin I and II synapses difficult as deficits to either the kinetics of exocytosis or the replenishment rate of the RRP could underlie the observations (Sun et al., 2006). Recent studies from immature Calyx of Held-MNTB synapses suggest that loss of synapsin I and II seem to selectively affect
the P_r of SVs released during the steady-state of high-frequency trains, but not the refilling rate of the
RRP (Sun et al., 2006). This raises the possibility that Sept5, binding to syntaxin proteins between SVs,
may function to “define” the RRP. In addition, this may provide an alternate primary role for Sept5 in
mature synapses besides in influencing the spatial coupling of VGCCs and SVs. Our data appears to
confirm that Sept5 has a role in “limiting” the size of the RRP throughout development.

Increases in the size of the RRP have been observed at the calyx of Held in response to tetanic
stimulation and have been hypothesized to underlie some forms synaptic plasticity at other synapses
(Byrne & Kandel, 1996; Rosenmund et al., 2002; Zhao & Klein, 2004; Habets & Borst, 2007). Also,
calmodulin has been implicated in the determination of RRP size in cultured hippocampal neurons
(Junge et al., 2004), suggesting a role for Ca^{2+} in signalling RRP plasticity. In addition, both Ca^{2+} and
cAMP have modulatory effects on a number of CAZ proteins such as; synapsin, Munc18, and RIM
(Calakos et al., 2004; Sun et al., 2006; Toonen et al., 2006). Sept5 may act in conjunction with any of
these proteins, through its GTPase activity, or independently, to regulate the number of SV accessible to
the release machinery, however, the precise mechanism of Sept5 action remains to be determined.
9.3 PROOF OF PRINCIPLE: SYNAPTIC DELAY & VGCC-SV COUPLING DISTANCE

9.3.1 Overview & Model Rationale.

In chapter 5 we provided experimental evidence that, given identical AP waveforms, mature synapses showed significantly shorter SD than did immature synapses (~200μs difference) (Fig. 5.6D). Here we attempt to further rationalize our previous hypothesis that this developmental reduction in SD is primarily derived from a spatial tightening of VGCCs and SVs. In particular, we have reproduced two existing models: the first describing buffered Ca\(^{2+}\) diffusion around a single VGCC and the second describing the properties of the Ca\(^{2+}\) sensor for release (Naraghi & Neher, 1997; Bollmann et al., 2000; Bollmann & Sakmann, 2005). Using experimental data acquired from mature synapses we then extended these models to hypothesize the release site topography in the mature synapse (Fedchyshyn & Wang, 2005; Yang & Wang, 2006). These models represent a simplified interpretation of release site topography which allows for simplification of the calculations required to evaluate the timing and efficacy of SV release. Despite these simplifications, the model reproduces many experimental observations; the validity of these assumptions will be discussed further below.

We began by reproducing the linearized buffered Ca\(^{2+}\) diffusion model of Naraghi and Neher (1997). Given the composition of Ca\(^{2+}\) buffers contained within a presynaptic recording pipette, this model allows for the calculation of the [Ca\(^{2+}\)] at any distance from the mouth of a single VGCC (Fig. 9.3.1). A particularly useful aspect of a linearized system is that it allows for the use of the superposition principle in accounting for greater numbers of VGCCs. Thus, the peak [Ca\(^{2+}\)] can be determined at an arbitrary distance (r) from any desired arrangement of VGCCs simply by summing their individual contributions. This then allows freedom to assume any distribution of VGCCs around a SV while being able to determine the [Ca\(^{2+}\)] at that point, which then provides a constraint governing the peak [Ca\(^{2+}\)] experienced by the release sensor for those particular set of buffer conditions.

With a means to determine [Ca\(^{2+}\)] at the release sensor, we then reproduced the kinetic release model of Bollmann et al. (2000) which accurately describes the release properties of the immature calyx of Held. We used a Gaussian distribution to approximate the [Ca\(^{2+}\)] profile as a function of time with the peak of the Gaussian limited to the [Ca\(^{2+}\)] described by the buffered Ca\(^{2+}\) diffusion model (as above). This [Ca\(^{2+}\)] profile was then used to drive the kinetic release model and determine the synaptic output and the kinetics of SV release under various conditions.

9.3.2 Buffered Diffusion Model.

As above, the buffered Ca\(^{2+}\) diffusion model describes the [Ca\(^{2+}\)] at some distance from a single VGCC under any set of buffer conditions (Fig. 9.3.1A). If the standing [Ca\(^{2+}\)] gradients are assumed to be at steady-state around an open VGCC, and buffer saturation is small, then the differential equations describing buffered Ca\(^{2+}\) diffusion can be linearized, considerably simplifying the mathematical description of the system (Neher, 1998; Naraghi & Neher, 1997). The small buffer saturation assumption has been shown to be valid, even for Ca\(^{2+}\) buffers with high binding ratios and short length constants like...
BAPTA, conservatively assuming single VGCC flux of around 0.3pA or smaller (Naraghi & Neher, 1997). In our simulations, this approximation should also hold as we have chosen single VGCC flux to be well below this threshold at 0.2pA.

At steady state, the $[Ca^{2+}]$ carried by three Ca$^{2+}$ buffers can be described by the following;

$$\frac{\delta B_1}{\delta B_{21}} = e^{-r\sqrt{C}} \cdot C^{-1} \varphi - C^{-1} \varphi \frac{r}{r}$$

$$\delta[Ca^{2+}] = \frac{\Phi}{4\pi D_{Ca}} - \sum_{j=1}^{3} \frac{D_j}{D_{Ca}} \delta B_j$$

Where $\Phi$ is the single VGCC flux and;

$$C = \left( \begin{array}{ccc}
\frac{1}{\tau_1 D_1} + \frac{\kappa_1}{\tau_1 D_{Ca}} & \frac{\kappa_1 D_2}{\tau_1 D_{Ca}} & \frac{\kappa_1 D_3}{\tau_1 D_{Ca}} \\
\frac{\kappa_2 D_1}{\tau_2 D_{Ca}} & \frac{1}{\tau_2 D_2} + \frac{\kappa_2}{\tau_2 D_{Ca}} & \frac{\kappa_2 D_3}{\tau_2 D_{Ca}} \\
\frac{\kappa_3 D_1}{\tau_3 D_{Ca}} & \frac{\kappa_3 D_2}{\tau_3 D_{Ca}} & \frac{1}{\tau_3 D_3}
\end{array} \right) \quad \varphi = \frac{\Phi}{4\pi D_{Ca}} \cdot \left( \begin{array}{c}
-\kappa_1 \\
-\kappa_2 \\
-\kappa_3
\end{array} \right)$$

$D_N$ is the diffusion coefficient of the $N^{th}$ buffer species and;

$$\kappa_N = \left( \frac{[B_N]}{[Ca^{2+}] + K_N} \right) \quad \tau_N = \frac{1}{k_{Non} + k_{Non} \cdot [Ca^{2+}]}$$

In these equations, $B_N$ is the concentration of the $N^{th}$ buffer species, $K_N$ is the dissociation constant of the $N^{th}$ buffer species, $k_{Non}$ is the Ca$^{2+}$ unbinding rate of the $N^{th}$ buffer species, and $k_{Non}$ is the Ca$^{2+}$ binding rate of the $N^{th}$ buffer species. The choices for the above parameters are shown in Table 9.3.1A and are taken directly from Naraghi & Neher (1997) with the exception of $\Phi$ which was taken as an average from Stanley (1993) and Shahrezaei & Delaney (2004). Given the necessary parameters, equation 9.2 can be solved to determine the $[Ca^{2+}]$ at any distance $r$ from a single VGCC for $N$ buffer species'.

Using the above equations, we have shown the $[Ca^{2+}]$ as a function of $r$ for various buffer compositions used throughout chapters 3-6 (Fig. 9.3.1B). In addition, Fig 9.3.1C shows $[Ca^{2+}] \cdot r$ as a function of $r$, which eliminates the $1/r$ dependence of $[Ca^{2+}]$ diffusion, and shows more clearly the characteristic length constant of the given buffer compositions (as in Naraghi & Neher, 1997). Note that the hypothesized endogenous mobile buffer properties, recently uncovered at the immature calyx of Held, have not been included here (Müller et al., 2007). This assumption is due to the fact that any
endogenous mobile buffers contained within the calyx of Held are likely dialysed rapidly with those contained in the much larger volume of the presynaptic patch pipette (see section 2.2.2).

To determine the $[\text{Ca}^{2+}]$ at the release sensor, we selected a simple ring of VGCCs, at equal $r$ from the SV, as our simplified topography. It is well established that, at least in the immature synapse, VGCCs are most likely at variable distances from their SV, located in clusters or some other heterogeneous spacing arrangement (Naraghi & Neher, 1997; Meinrenken et al., 2002; Meinrenken et al., 2003). However, assuming a more simplistic arrangement of a ring of VGCCs simplifies the determination of $[\text{Ca}^{2+}]$ arising from multiple VGCCs and, as we will show, sufficiently address whether VGCC-SV coupling differences can account for developmental changes in SD.

Previous modeling studies, performed with data acquired from the immature synapse, have hypothesized an average VGCC-SV separation of ~80nm (Meinrenken et al., 2002). In addition, morphological data, at least from the immature synapse, can provide some constraints when choosing and testing potential VGCC-SV separation distances. AZs at the immature calyx of Held are approximately $0.01\mu\text{m}^2$ in area (Sätzler et al., 2002). If we assume that the AZ is approximately circular in shape, then the diameter of the AZ is ~120nm and allows for two docked SVs on average (Sätzler et al., 2002, Taschenberger et al., 2002). This approximate AZ geometry implies that VGCCs can be separated by no more than ~100nm from any given SV, assuming VGCCs are in the AZ, thus providing an upper limit on the testing ranges for VGCC-SV separation. Similarly, the minimum separation distance between SVs and VGCCs is limited by the presence of the release machinery, and its many associated proteins, at the base of the SV. The SNARE complex (ring) is estimated to have a diameter of ~20nm or larger depending on the size of the SV (Cho et al., 2005), thus we considered 20nm to be a lower limit for VGCC-SV separation in our modelled SV arrangements.

Given these limits, we tested a number of possible VGCC-SV separation distances with different numbers of VGCCs contributing to the $[\text{Ca}^{2+}]$ transient in each case. For the immature synapse, we found that a ring of 12 VGCCs at a distance of $r=61$nm reproduced experimental data well while remaining in line with the average spacing determined by Meinrenken et al. (2002).

Upon invasion of an immature AP into the calyx of Held, approximately 50% of VGCCs open (Yang & Wang, 2006). Therefore, we summated the $[\text{Ca}^{2+}]$ contribution from 6 of the 12 VGCCs and set this as the peak $[\text{Ca}^{2+}]$ for the input into the kinetic release model. Note that the number of VGCCs activated (i.e. 6) is in line with our measured Ca$^{2+}$-domain cooperativity values from immature synapses (Fedchyshyn & Wang, 2005; $m=5-6$).

Knowing that VGCC-SV coupling tightens in the mature synapse and knowing that fewer VGCCs are required to release a SV (Fedchyshyn & Wang, 2005), we hypothesized a different topography of VGCCs than that of the immature synapse. In the mature synapse, we found that a ring of 9 VGCCs at a distance of $r=29$nm reproduced our experimental findings well. In response to the narrower mature APs, approximately 35% of VGCCs open (Yang & Wang, 2006). As a result we summated the contribution of 3 VGCCs as the peak $[\text{Ca}^{2+}]$ at the SV for input into the kinetic release model. Note that 3 open VGCCs is also in line with our measured cooperativity values for this developmental stage (Fedchyshyn & Wang, 2005; $m=\sim3$). The above arrangements are illustrated schematically in Fig. 9.3.1D with parameters.
Figure 9.3.1: Linearized Buffered Ca\(^{2+}\) Diffusion & Release Site Topography Model. 
(A) Schematic representation of the single VGCC for which the buffered diffusion model determines [Ca\(^{2+}\)] as a function of r. (B) [Ca\(^{2+}\)] as a function of r for the various buffer conditions from Fedchyshyn & Wang (2005) in response to the steady-state opening of a single VGCC. (C) [Ca\(^{2+}\)]\cdot r as a function of r, or the buffer composition “fingerprint” for the various buffer conditions from Fedchyshyn & Wang (2005) in response to the steady-state opening of a single VGCC. (D) Modeled arrangement of VGCCs in immature (magenta VGCCs) and mature (cyan VGCCs) synapses. ENDO refers to endogenous immobile buffer. Single VGCC flux for all panels \(\Phi = 6.24 \times 10^5\) ions/sec or 0.2pA of constant current. Buffer parameters as in Naraghi & Neher (1997). Schematics not drawn to scale.
shown in the adjacent table. All model calculations were coded and performed in Maple11 (Maplesoft, Waterloo Maple).

### 9.3.3 Kinetic Release Model.

Our description of the release sensor is based on the model of Bollmann et al. (2000) which accurately describes SV release at the immature calyx of Held. More recently, the authors have shown their model to be robust even when \([\text{Ca}^{2+}]\) transients resemble those evoked by APs (Bollmann & Sakmann, 2005). The sequential scheme (Fig. 9.3.2A) can be written as a system of 8 first order differential equations, describing the rate of change of each of the schemes species, and one equation describing the \([\text{Ca}^{2+}]\) transient as a function of time;

\[
\frac{dSV(t)}{dt} = -5k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SV(t) + k_{off} \cdot SVCa(t) \quad (9.3)
\]

\[
\frac{dSVCa_1(t)}{dt} = -4k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_1(t) + 2k_{off} \cdot SVCa_2(t) + 5k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SV(t) - k_{off} \cdot SVCa_1(t) \quad (9.4)
\]

\[
\frac{dSVCa_2(t)}{dt} = -3k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_2(t) + 3k_{off} \cdot SVCa_3(t) + 4k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_1(t) - 2k_{off} \cdot SVCa_2(t) \quad (9.5)
\]

\[
\frac{dSVCa_3(t)}{dt} = -2k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_3(t) + 4k_{off} \cdot SVCa_4(t) + 3k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_2(t) - 3k_{off} \cdot SVCa_3(t) \quad (9.6)
\]

\[
\frac{dSVCa_4(t)}{dt} = -k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_4(t) + 5k_{off} \cdot SVCa_5(t) + 2k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_3(t) - 4k_{off} \cdot SVCa_4(t) \quad (9.7)
\]

\[
\frac{dSVCa_5(t)}{dt} = -\gamma \cdot SVCa_5(t) + \delta \cdot SVCa_5(t) + k_{on} \cdot [\text{Ca}^{2+}]_t \cdot SVCa_4(t) - 5k_{off} \cdot SVCa_5(t) \quad (9.8)
\]

\[
\frac{dSVCa^*_5(t)}{dt} = -\rho \cdot SVCa^*_5(t) + \gamma \cdot SVCa_5(t) - \delta \cdot SVCa^*_5(t) \quad (9.9)
\]

\[
\frac{dF(t)}{dt} = \rho \cdot SVCa^*_5(t) \quad (9.10)
\]
where the various model parameters are listed in Table 9.3.1B. To describe $[\text{Ca}^{2+}]_v(t)$ we use a Gaussian to approximate the $[\text{Ca}^{2+}]$ transient experienced by the release sensor. The AP-evoked $[\text{Ca}^{2+}]$ transient closely resembles a Gaussian distribution with a width that is similar to that of the whole-cell $I_{\text{Ca}}$ (Meinrenken et al., 2002; Bollmann & Sakmann, 2005).

$$[\text{Ca}^{2+}]_v(t) = I_{\text{Ca peak}} \cdot e^{-\frac{1}{2} \left( \frac{t - t_0}{\sigma} \right)^2} + C_{\text{rest}}$$ (9.11)

Under our typical experimental conditions, we measure $I_{\text{Ca}}$ half-widths of ~300$\mu$s and ~200$\mu$s for immature and mature synapses respectively, in response to their native APs (Yang & Wang, 2006). Thus the half-width of the Gaussian $[\text{Ca}^{2+}]$ transient ($\sigma$) was set to these values depending on which AP type was being simulated. The peak of the Gaussian was determined by the steady-state $[\text{Ca}^{2+}]$ at the centre of the ring of channels as described above. The peak of the Gaussian was established as $t=0$, unless otherwise stated. It should be noted that only the peak $[\text{Ca}^{2+}]$ of the $[\text{Ca}^{2+}]$ transient is strongly affected by the distance between VGCCs and SVs while the half-width depends primarily on the width of the AP (Meinrenken et al., 2002).

For both immature and mature synapses we defined a pool of release sensors that had docked SVs present, this quantity being conceptually distinct from the traditionally defined RRP in that these are all of the possible SVs available to a single AP. In immature and matures synapses, 2000 and 3000 docked SVs were used in simulations respectively, in accordance with morphological and electrophysiological data at these developmental stages (Sätzler et al., 2002; Taschenberger et al., 2002). The above system of equations was then solved numerically using the fourth-fifth order Runge-Kutta method in Maple11 (Maplesoft, Waterloo Maple).

### 9.3.4 Model Output – AP in Immature & Mature Synapses.

We first tested the above models using our standard patch pipette buffering conditions (2mM ATP, 0.5mM ENDO, & 0.5mM EGTA). In this simulation we used the AP$_1$ waveform to drive $I_{\text{Ca}}$ in both immature and mature synapses which effectively opens ~50% of VGCCs in either synapse (Yang & Wang, 2006). In immature synapses at $r=61$nm, peak $[\text{Ca}^{2+}]$ was found to be 13.8$\mu$M when 6 VGCCs open. For mature synapses at $r=29$nm, peak $[\text{Ca}^{2+}]$ was 28$\mu$M when 4.5 VGCCs open. These values were then used to constrain the peaks of the respective $[\text{Ca}^{2+}]$ transients in simulated immature and mature synapses. Since AP$_1$ waveforms were being used in both immature and mature synapses, $[\text{Ca}^{2+}]$ transient widths were maintained at 300$\mu$s (Fig. 9.3.2B(i), left panel).

As expected, the larger mature $[\text{Ca}^{2+}]$ transient produced a significantly higher peak release rate than in the immature synapse (2942SV/ms vs. 490SV/ms) (Fig. 9.3.2B(i), center panel). Based on the docked SV pools defined for immature and mature synapses, we calculated the cumulative number of SVs released as a function of time (Fig. 9.3.2B(i), right panel). Immature synapses are expected to release ~10% of docked SVs in response to a single AP$_1$ waveform while mature synapses are expected to release ~45% of docked SVs in response to the same waveform (Yang & Wang, 2006). In response
to the simulated \([Ca^{2+}]\) transients, immature synapses released 222 SVs, which is 11.1% of the docked pool of SV, as expected and in line with other reports (Sakaba & Neher, 2001; Borst & Sakmann, 1996). Likewise, mature synapses released 1414 SVs in response to the same AP which represents 47% of the docked SV pool. Peak release rates are shown as a function of peak \([Ca^{2+}]\) for both immature and mature synapses, in response to their native APs, in Fig. 9.3.2D.

When we measured the delay in the response of the simulated release sensors in response to the immature and mature \([Ca^{2+}]\) transients, the maximal release rate was reached only 30μs following the peak of the mature \([Ca^{2+}]\) transient and 140μs following the peak of the immature \([Ca^{2+}]\) transient. This represents a difference of 110μs due solely to the differential response of the \(Ca^{2+}\) sensor to the different \([Ca^{2+}]\) transients. This delay does not include potential differences in the time required for diffusion of \(Ca^{2+}\) or in establishment of the equilibrium \([Ca^{2+}]\) gradient (discussed below).

These data suggest that, of the ~200μs difference in SD observed between immature and mature synapses in response to the AP, waveforms, over 50% of it can be accounted for solely by the kinetics of the release sensor (see Fig. 5.6D). This is in agreement with previous findings (Bollmann et al., 2000; Schneggenburger & Neher, 2000; Meinrenken et al., 2002; Felmy et al., 2003; Bollmann & Sakmann, 2005). The dependence of delay as a function of peak \([Ca^{2+}]\) can be seen in Fig. 9.3.2D for both immature and mature synapses in response to their native APs.

### 9.3.5 Model Output – Immature Synapses With & Without 10mM EGTA.

In chapter 3 we demonstrated a developmental decrease in the effectiveness of 10mM EGTA at attenuating SV release when loaded presynaptically. Using our simplified model, we sought to test this result in the immature synapse. When EGTA was loaded presynaptically into immature synapses, a reduction of ~60% was observed (Fig. 3.2). Using the buffered \(Ca^{2+}\) diffusion model we increased the \([EGTA]\) to 10mM and determined the \([Ca^{2+}]\) at a distance of 61nm from 6 open VGCCs (Fig. 9.3.2B(ii), left panel). Under these conditions, \([Ca^{2+}]\) was found to be 10μM. Using a \([Ca^{2+}]\) transient with a 300μs width and 10μM peak, the release model showed a significantly reduced maximal release rate (195SV/ms vs. 490SV/ms, 0.5mM & 10mM EGTA respectively) (Fig. 9.3.2B(ii), center panel). This EGTA-mediated reduction in release rate reduced the total number of released SVs to 86, a 61% reduction (Fig. 9.3.2B(ii), right panel). Interestingly, the model also accurately predicts the slight (~25%) decrease in SV release when mature synapses are loaded with 10mM EGTA (data not shown). Not surprisingly, the model cannot account for the differential effects of both EGTA and BAPTA in either mature or immature synapses (data not shown), which requires the addition of VGCC positional heterogeneity (Naraghi & Neher, 1997; Meinrenken et al., 2002).

### 9.3.6 Model Output – Immature & Mature Synapses With Native AP Waveforms.

In a final scenario, we used native \([Ca^{2+}]\) transients to drive simulated immature and mature release sensors. In this scheme, we aligned \([Ca^{2+}]\) transients by their 5% rise time in order to estimate the substantial change in delay observed between immature and mature synapses in response to
Figure 9.3.2: Kinetic Release Model Output for Simulated Immature & Mature Synapses.

(A) Kinetic reaction scheme for 5-site sequential Ca$^{2+}$ binding model of SV release. (B) Release model output for the following scenarios. (i) Ca$^{2+}$ input function (left panel), release rate (center panel), and total SVs released (right panel) as a function of time for simulated immature (black) and mature (red) synapses. (ii) As in (i) for simulated immature synapses with 0.5mM EGTA (black) and 10mM EGTA (blue) in the presynaptic patch pipette and in response to the AP waveform. (iii) As in (i) using an AP$^I$ waveform in a simulated immature synapse (black) and an AP$_M$ waveform in a simulated mature synapse (green). Note that in (i) and (ii), Ca$^{2+}$ input functions are aligned by their peaks at t=0 while in (iii) the 5% rise of Ca$^{2+}$ input functions is used for alignment. Vertical dotted lines in center panels refer to the time of maximal release rate. (C) Peak release rates as a function of peak [Ca$^{2+}$] for the conditions shown in B(iii). (D) Delay to maximum release rate, measured from peak of respective [Ca$^{2+}$] inputs, as a function of peak [Ca$^{2+}$] for the conditions shown in C. AP$_m$ denotes AP waveform simulated by Gaussian [Ca$^{2+}$] (t)
evoked stimulation (Taschenberger & von Gersdorff, 2000). We retained the peak of the immature \([\text{Ca}^{2+}]\) transient as \(t=0\) while shifting the mature \([\text{Ca}^{2+}]\) transient earlier in time by \(\sim 250 \mu s\) (i.e. \(t=-0.250 \mu s\)) to simulate the difference in delay following the arrival of either AP (Fig. 9.3.2B(iii), left panel). Peak release rates were 490SV/ms and 1041SV/ms for immature and mature synapses respectively with the total delay difference being \(\sim 360 \mu s\) (Fig. 9.3.2B(iii), center panel). Immature synapses again released 222 SVs in response to their native \([\text{Ca}^{2+}]\) transient while mature synapses release 329 SVs in response to their native \([\text{Ca}^{2+}]\) transient (Fig. 9.3.2B(iii), right panel). This amount of release represents \(\sim 11\%\) of the mature synapses pool of docked SVs as expected based on experimental observation at this developmental stage (Yang & Wang, 2006).

These data suggest that by accounting for both the delay in the onset of the \([\text{Ca}^{2+}]\) transient and the delayed response of the \(\text{Ca}^{2+}\) sensor, much of the \(\sim 400 \mu s\) difference in SD observed between immature and mature synapses in response to evoked APs can be explained through the positioning of VGCCs relative to SVs (Taschenberger & von Gersdorff, 2000, see chapter 5).

### 9.3.7 Additional Sources of Presynaptic Delay.

Although we can account for over 50\% of the observed difference in SD between immature and mature synapses (AP, waveform, Fig. 5.6) by simply considering a tightening in the VGCC-SV coupling, the question remains whether we can rationalize the remaining delay in the same way?

Our simplified model of VGCC-SV coupling exclusively determines the component of overall delay that resides downstream of the establishment of steady-state \([\text{Ca}^{2+}]\) gradients. In reality, these gradients take a finite time to stabilize which depends on the distance from the open VGCC (i.e. diffusion). Naraghi & Neher (1997) have estimated the time required for stability of \([\text{Ca}^{2+}]\) in the presence of 2mM EGTA-like buffer. At a distance of 60nm, \([\text{Ca}^{2+}]\) gradients require approximately 100\(\mu\)s to reach 90\% stability while at 30nm 90\% stability is reached in approximately 50\(\mu\)s. Thus, it is likely that this upstream factor comprises an additional portion of the observed delay difference between immature and mature synapses and also depends on the VGCC-SV coupling. The \(\text{Ca}^{2+}\) sensor therefore experiences a much “sharper” \([\text{Ca}^{2+}]\) transient in mature synapses which may not be adequately reproduced by simply narrowing the half-width of the Gaussian input. As above, this could account for a further 50\(\mu\)s of presynaptic delay.

In addition, the properties of the release sensor could change with development which may alter its kinetic response to a given \([\text{Ca}^{2+}]\) transient. If the sensitivity of the \(\text{Ca}^{2+}\) sensor were to increase with development, shortening of SD could occur presynaptically independent of VGCC-SV coupling. However, our recent findings suggest that the mature \(\text{Ca}^{2+}\) sensor is less sensitive than that of the immature synapses by a factor of \(\sim 2\) (Wang, Neher & Taschenberger, unpublished observations). Given these preliminary findings, it is unlikely that alterations to the properties of the \(\text{Ca}^{2+}\) sensor account for the observed developmental shortening in SD.
### A

**Buffered Ca\(^{2+}\)** Diffusion Model:

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Immature (P8-12)</th>
<th>Mature (P16-18)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP Diffusion Constant</td>
<td>(220 \mu m^2/s)</td>
<td>(220 \mu m^2/s)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(K_a (ATP))</td>
<td>(2300 \mu M)</td>
<td>(2300 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(k_{on} (ATP))</td>
<td>(500 \mu M \cdot s^{-1})</td>
<td>(500 \mu M \cdot s^{-1})</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>([ATP]_{pipette})</td>
<td>(2000 \mu M)</td>
<td>(2000 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>EGTA Diffusion Constant</td>
<td>(220 \mu m^2/s)</td>
<td>(220 \mu m^2/s)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(K_a (EGTA))</td>
<td>(0.18 \mu M)</td>
<td>(0.18 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(k_{on} [EGTA])</td>
<td>(2.5 \mu M \cdot s^{-1})</td>
<td>(2.5 \mu M \cdot s^{-1})</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>([EGTA]_{pipette})</td>
<td>(500/10000 \mu M)</td>
<td>(500 \mu M)</td>
<td>Fedchyshyn &amp; Wang, 2005</td>
</tr>
<tr>
<td>BAPTA Diffusion Constant</td>
<td>(220 \mu m^2/s)</td>
<td>(220 \mu m^2/s)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(K_a (BAPTA))</td>
<td>(0.22 \mu M)</td>
<td>(0.22 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(k_{on} (BAPTA))</td>
<td>(400 \mu M \cdot s^{-1})</td>
<td>(400 \mu M \cdot s^{-1})</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>([BAPTA]_{pipette})</td>
<td>(1000 \mu M)</td>
<td>(1000 \mu M)</td>
<td>Fedchyshyn &amp; Wang, 2005</td>
</tr>
<tr>
<td>ENDO Diffusion Constant</td>
<td>(15 \mu m^2/s)</td>
<td>(15 \mu m^2/s)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(K_a (ENDO))</td>
<td>(50 \mu M)</td>
<td>(50 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>(k_{on} (ENDO))</td>
<td>(100 \mu M \cdot s^{-1})</td>
<td>(100 \mu M \cdot s^{-1})</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>([ENDO]_{pipette})</td>
<td>(500 \mu M)</td>
<td>(500 \mu M)</td>
<td>Naraghi &amp; Neher, 1997</td>
</tr>
<tr>
<td>Ca(^{2+}) Diffusion Constant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting ([Ca^{2+}])</td>
<td>(50nM)</td>
<td>(50nM)</td>
<td>Helmhchen et al., 1997</td>
</tr>
<tr>
<td>Steady-State Single VGCC Flux</td>
<td>(6.24 \times 10^4 ions/s)</td>
<td>(6.24 \times 10^4 ions/s)</td>
<td>Stanley, 1993</td>
</tr>
</tbody>
</table>

### B

**Kinetic Release Model:**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Immature (P8-12)</th>
<th>Mature (P16-18)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{on})</td>
<td>(0.24 \mu M \cdot ms^{-1})</td>
<td>(0.24 \mu M \cdot ms^{-1})</td>
<td>Bollmann &amp; Sakmann, 2005</td>
</tr>
<tr>
<td>(k_{off})</td>
<td>(3 ms^{-1})</td>
<td>(3 ms^{-1})</td>
<td>Bollmann et al., 2000</td>
</tr>
<tr>
<td>(\delta)</td>
<td>(30 ms^{-1})</td>
<td>(30 ms^{-1})</td>
<td>Bollmann et al., 2000</td>
</tr>
<tr>
<td>(\gamma)</td>
<td>(8 ms^{-1})</td>
<td>(8 ms^{-1})</td>
<td>Bollmann et al., 2000</td>
</tr>
<tr>
<td>(\rho)</td>
<td>(40 ms^{-1})</td>
<td>(40 ms^{-1})</td>
<td>Bollmann et al., 2000</td>
</tr>
<tr>
<td>Peak ([Ca^{2+}]), (<a href="t">Ca^{2+}</a>) half-width</td>
<td>Determined from Buffer Model</td>
<td>Determined from Buffer Model</td>
<td></td>
</tr>
<tr>
<td>(<a href="t">Ca^{2+}</a>) half-width</td>
<td>(0.3 ms)</td>
<td>(0.2 ms)</td>
<td>Yang &amp; Wang, 2006</td>
</tr>
<tr>
<td>RRP Size</td>
<td>(2000)</td>
<td>(3000)</td>
<td>Taschenberger et al., 2002</td>
</tr>
<tr>
<td>Number of VGCCs/SV (open/total)</td>
<td>(6/12)</td>
<td>(3/9)</td>
<td>Fedchyshyn &amp; Wang, 2005</td>
</tr>
<tr>
<td>Separation Distance</td>
<td>(61 nm)</td>
<td>(29 nm)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 9.3.1:** Parameters For Buffered Ca\(^{2+}\) Diffusion & Kinetic Release Models:

(A) Table of parameters used to construct the buffered Ca\(^{2+}\) diffusion model. (B) Parameters used in the kinetic release model.
9.3.8 Sources of Postsynaptic Delay.

While we have focused on sources of presynaptic delay, a number of mechanisms downstream of SV release may account for a component of developmental shortening in SD. For example, a decrease in the diffusion distance for glutamate within the synaptic cleft can alter the time course of AMPAR activation and thus the delay measured to the $I_{\text{EPSC}}$ peak (Trommershäuser et al., 1999). However, delay stemming from altering the AMPAR-release site distances by as much as 350nm results in only a $\sim 100\mu s$ change in the delay to peak release rate (Trommershäuser et al., 1999). In these same simulations, the effect on the onset of release was significantly more subtle. Our measurement of SD is taken at the onset of $I_{\text{EPSC}}$, suggesting that we are measuring SD by the SVs that most rapidly activate AMPARs. Thus it is likely that reorganization of AMPARs, relative to the SV release sites, contributes minimally to our measurement of SD, but may contribute more to measures using $I_{\text{EPSC}}$ peak as a reference (i.e. RRD) (Hermida et al., 2006).

Another potential source of postsynaptic delay changes are developmental changes in the activation kinetics of the AMPARs themselves. However, it is hypothesized that the reduction in the rise time of $I_{\text{EPSC}}$ with development is the result of improved synchrony of SV release rather than from the more rapid kinetics of AMPARs (Taschenberger & von Gersdorff, 2000). As above, our determination of SD, using the onset of $I_{\text{EPSC}}$, is resistant to the significant impact asynchrony has on the rise time of $I_{\text{EPSC}}$ (Fedchyshyn & Wang, 2007).

9.3.9 General Discussion & Overview.

Above we have taken a simplified model of the calyx of Held and extended it to provide proof of principle that differences in SD, observed between immature and mature synapses, can be explained by a change in coupling between VGCCs and SVs. While the release site topography has not been previously modelled in the mature synapse, our simplified topography in the immature synapses agrees well with those previously published in this system (Meinrenken et al., 2002). In addition, the peak $[\text{Ca}^{2+}]$ experienced by the simulated release sensor are in line with the measured sensitivity of the immature Ca$^{2+}$ sensor for all tested conditions (Bollmann et al., 2000; Schneggenburger & Neher, 2000).

Besides reproducing experimental data, as above, the kinetic release model is able to predict a difference in the dependence of delay on peak $[\text{Ca}^{2+}]$ between immature and mature synapses as observed in chapter 5 (Fig. 5.3, 9.3.2D). In addition, the model predicts that the range of delays possible in the mature synapse is significantly smaller than that in the immature synapse which is again in agreement with our findings in chapter 5. As described in chapter 7, the discrepancy in the shapes of the $[\text{Ca}^{2+}]$-SD relationship (i.e. linear vs. exponential) likely due to the small range of $[\text{Ca}^{2+}]$ sampled in the experiments of chapter 5. Regardless, this interesting developmental difference is accounted for by simply considering developmental differences in VGCC-SV coupling.

It should also be noted that, under conditions of very high peak $[\text{Ca}^{2+}]$ (>50$\mu$M in the immature synapse), the peak release rate can actually occur prior to the peak of the $[\text{Ca}^{2+}]$ transient similar to the result of Sabatini & Regehr (1999) (Fig. 9.3.2D). However, at peak $[\text{Ca}^{2+}]$ of this magnitude, single $[\text{Ca}^{2+}]$ transients are sufficient to deplete a majority of the docked SV pool under our conditions. Release
probabilities of this magnitude have not been observed at the calyx of Held to date but may occur at other synapses. Therefore, at reasonable [Ca$^{2+}$], release rate follows the shape of the [Ca$^{2+}$] transient but at a slight delay which depends on the magnitude of the transient (Meinrenken et al., 2002).

In summary, the above simplified model demonstrates that, under conditions of identical input (ie. AP$_i$), a decrease in the distance between VGCCs and SVs underlies a significant portion of the observed developmental decrease in SD.