NMDAR-Dependent Synaptic Plasticity at the Calyx of Held Synapse

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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2012

ABSTRACT

NMDARs are indispensable for developmental plasticity in the mammalian brain, but their roles \textit{in vivo} are difficult to ascertain as NMDAR-knockouts are lethal. To circumvent this problem, we utilized NR1-knockdown mice to examine plasticity at the calyx of Held-MNTB synapse in the auditory brainstem. Previous work shows NMDARs at this synapse are rapidly down-regulated following the onset of hearing (P12), leading to the hypothesis that transient NMDAR expression is indispensable for modulating functional and morphological remodelling during development. We tested this by performing electrophysiological recordings, fluorescence tracing \textit{in vitro}, and auditory brainstem responses \textit{in vivo}, and surprisingly found that reducing postsynaptic NMDARs appears to promote functional maturation via presynaptic mechanisms in the absence of morphological and acoustic transmission differences in the mature calyx. This suggests a novel role for NMDARs to function as an activity-dependent control for setting the pace of consolidation and maturation in the calyx of Held synapse.
ACKNOWLEDGEMENTS

Special thanks to my supervisor, Dr. Lu-Yang Wang, whose mentorship and friendship throughout the development of this project has allowed me to significantly enhance my research and critical thinking skills. He has provided me with every opportunity to grow as a scientist and has introduced me to several great thinkers to expand my intellect. His drive and enthusiasm has truly been a positive influence both professionally and personally. I have been very lucky to find a supervisor who is extremely dedicated to the advancement of his students and staff.

This project would not have come together if not for the significant contributions of my friends and fellow members of the Wang lab, specifically: Dr. Giovanbattista Grande who embedded the fluorescent crystals for imaging studies and performed the in vivo auditory brainstem recordings; Jamila Aitoubah who bred and promptly genotyped the mouse litters at a much younger age than she is used to and allowed me perform my experiments as early as postnatal day 9; Dr. Yi-Mei Yang who greatly aided the development of my recording techniques and helped perform initial experiments when I was still inefficient; and Dr. Laurence David, Stephen Lesperance, Derek Howard, and Dr. Myriam Lafreniere-Roula whose support, encouragement and suggestions have made my experience in the lab extremely rewarding. Their willingness to discuss their experiences has provided me with great insights on how to develop my career in research and science.

I would also like to thank Dr. Amy Ramsey who developed the NR1-KD mice that have been a crucial tool in this project. Her generous donation of the mouse parents and advice on how to ensure the survival of the litters was instrumental in developing the project from the beginning. I also greatly appreciate the generosity and patience of Dr. Robert Harrison who graciously allowed us to use his equipment for the auditory
brainstem response recordings throughout the study.

Thank you to my supervisory committee members, Dr. Michael Salter and Dr. Zhengping Jia. Their support, guidance, and thought-provoking comments throughout my graduate education has significantly contributed to the project and forced me to question the motive and limitations for every experiment I performed and to acknowledge alternate explanations for every observation I made.

I also wish to acknowledge the University of Toronto and Sick Kids Hospital for providing me with a stimulating environment and invaluable peers and mentors, as well as acknowledge financial support from the Ontario Graduate Scholarship.

Finally, I must thank my family for their continued love and support throughout all my endeavours. I have been privileged to have two older sisters in research whose guidance and knowledge of the field has greatly influenced my education and personal decisions and has shaped my passion for science. Without all these individuals, I would not be the person I am today. Thank you so much.
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<tbody>
<tr>
<td>ABR</td>
<td>auditory brainstem responses</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid receptor</td>
</tr>
<tr>
<td>AMPAR-EPSC</td>
<td>excitatory postsynaptic current mediated by AMPA receptors</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APV</td>
<td>(2R)-amino-5-phosphonovaleric acid; NMDAR antagonist</td>
</tr>
<tr>
<td>aVCN</td>
<td>anterior ventral cochlear nucleus</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ions</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calmodulin kinase 2</td>
</tr>
<tr>
<td>CB1-R</td>
<td>type 1 cannabinoid receptors</td>
</tr>
<tr>
<td>CTZ</td>
<td>cyclothiazide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole stain</td>
</tr>
<tr>
<td>Dil</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate lipophilic dye</td>
</tr>
<tr>
<td>EGTA</td>
<td>calcium ion buffer, ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>gamma-aminobutyric acid receptor B</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>I&lt;sub&gt;-40&lt;/sub&gt; and I&lt;sub&gt;+40&lt;/sub&gt;</td>
<td>current at holding potential –40 mV and +40 mV, respectively</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ions</td>
</tr>
<tr>
<td>KS-test</td>
<td>Kolmogorov-Smirnov test of significance</td>
</tr>
<tr>
<td>LSO</td>
<td>lateral superior olive</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ions</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NG2 glia</td>
<td>neuron-glial antigen 2-positive glia</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NMDAR-EPSC</td>
<td>excitatory postsynaptic current mediated by NMDA receptors</td>
</tr>
<tr>
<td>NR1-KD</td>
<td>NR1 knockdown</td>
</tr>
<tr>
<td>NT</td>
<td>neurotransmitter</td>
</tr>
<tr>
<td>MEPSC</td>
<td>miniature/spontaneous excitatory postsynaptic current</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MNTB</td>
<td>medial nucleus of the trapezoid body</td>
</tr>
<tr>
<td>MSO</td>
<td>medial superior olive</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day; followed by a number</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PPD</td>
<td>paired-pulse depression</td>
</tr>
<tr>
<td>PPR</td>
<td>paired-pulse ratio</td>
</tr>
<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>RRP</td>
<td>readily releasable pool</td>
</tr>
<tr>
<td>SOC</td>
<td>superior olivary complex</td>
</tr>
<tr>
<td>STD</td>
<td>short-term depression</td>
</tr>
<tr>
<td>SV</td>
<td>synaptic vesicle</td>
</tr>
<tr>
<td>$\tau_{\text{fast}}$ and $\tau_{\text{slow}}$</td>
<td>fast and slow time constants, respectively</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channels</td>
</tr>
<tr>
<td>$V_h$</td>
<td>holding potential</td>
</tr>
<tr>
<td>$V_m$</td>
<td>membrane potential</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
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CHAPTER ONE: Background

1.1 Overview of Neuroplasticity in the Developing Brain

One of the most remarkable features of the brain is its ability to elicit changes in the wiring of the nervous system during development, learning, and adaptation to injury. This property, referred to as neuroplasticity, is particularly prominent during early development when experience has profound impact on functional adaptations that range from subcellular plasticity to full cortical remapping. Developmental plasticity involves modifications in neurons and their connections, known as synapses that form the building blocks of the nervous system and prime the brain network for efficient relay of neuronal signals (i.e. synaptic transmission), reliable computation and information storage. Most neural connections mature during a critical period of hypersensitivity to exogenous and endogenous factors. These factors are tightly controlled during that critical period of development to produce a highly specialized synapse for the function of that particular network. This thesis is aimed to probe the fundamental factors that gate functional and morphological remodelling using our model synapse: the mouse calyx of Held and the principal neuron synapse, located in the medial nucleus of the trapezoid body (MNTB) of the auditory brainstem, within the first three postnatal weeks when significant developmental changes take place in response to the onset of external auditory experience.

1.1.1 Synaptic Transmission and Plasticity

The synapse is a specialized structure that allows for the relay of either excitatory or inhibitory communication from one neuron to another. It is the basis for all neural processing, therefore its plasticity has the ability to either consolidate or prune...
connections, thus drastically altering behaviour and perception (Rayport & Kandel, 1986; Skelton, Miller & Phillips, 1985). The strength of this neuronal relay can be modulated at various stages of synaptic transmission. At the presynaptic terminal, an invading action potential (AP) activates voltage-gated calcium channels (VGCCs), which cause an influx of calcium ions (Ca\textsuperscript{2+}) that drive synaptic vesicle (SV) fusion and neurotransmitter release. This process can either be potentiated or depressed by altering the shape of the invading action potential through the expression of AP-driving voltage gated potassium (K\textsuperscript{+}) and sodium (Na\textsuperscript{+}) channels with different kinetics (Geiger & Jonas, 2000), the number of active zones that contain SV release machinery, the type of VGCCs, the proximity of the Ca\textsuperscript{2+} channels to SVs through interactions with scaffolding proteins such as septins (Fedchyshyn & Wang, 2005; Yang et al., 2010), the uptake of residual Ca\textsuperscript{2+}, the priming of SVs to the site of fusion, and the amount of neurotransmitter in each SV (Wang et al., 2011).

The neurotransmitter released in the synaptic cleft must be sensed by postsynaptic receptors in order to relay the information. In order to strengthen the relay, receptors are clustered into postsynaptic densities (PSDs) via tethering proteins (PSD-95, Homer1a-SHANK; Soria Van Hoeve & Borst, 2010, neuronal pentraxins; Xu et al., 2003) and are closely localized to presynaptic active zones through linkage proteins (neuroligins and neurexins; Graf et al., 2004). The signal can be potentiated or depressed at the postsynaptic site by modulating the type and number of receptors, whose current drives the action potential generation of the postsynaptic neuron. These modulations occur throughout the synapse which can either be transiently evoked (short-term plasticity) through activity or adjust and develop the synapse’s long-term firing properties (long-term plasticity).
1.2 Glutamatergic Synapses

The glutamatergic synapse is the predominant fast excitatory synapse in the mammalian central nervous system and is extremely well known for high sensitivity to modulation through various mechanisms of synaptic plasticity. It is one of the most studied synapses and has highly organised presynaptic and postsynaptic components for optimised synaptic signalling.

The glutamatergic synapse has a presynaptic terminal with synaptic vesicles loaded with the amino acid neurotransmitter L-glutamate (hereafter referred to as glutamate), which is released into the synaptic cleft upon presynaptic membrane depolarization and binds to postsynaptic glutamate receptors. The amount of glutamate released is highly dependent on several factors: the number of SVs docked in the presynaptic terminal; the quantal amount of glutamate in each SV; and the probability of synaptic vesicle fusion, influenced by sensitivity to presynaptic influx of Ca\(^{2+}\) and second messengers (reviewed in Conti & Weinberg, 1999). In initial sensory processing synapses in the brainstem, a single presynaptic AP can induce the fusion of multiple synaptic vesicles (low probability of release per vesicle) with varying degrees of synchrony (von Gersdorff \textit{et al.}, 1998). However, axo-spinal synapses, such as synapses in the cortex, are likely to release a single SV in response to an AP (reviewed in Walmsley, Alvarez & Fyffe, 1998).

The presynaptic release of neurotransmitter is only half the process of synaptic transmission. The binding of glutamate to receptors in the postsynaptic terminal is vital for the continuation of the signal, and the properties of the cleft in between the two terminals can mediate synaptic transmission. Glutamate spreads through the cleft at a rate dependent on cleft geometry and the composition of glycoproteins that increases viscosity of the cleft space and impedes diffusion. The spatiotemporal patterns of
glutamate release into the synaptic cleft and its clearance by neighbouring astrocytes highly affect its binding to postsynaptic glutamate receptors, and the resulting excitatory postsynaptic currents (EPSCs).

Released by the presynaptic terminal into the synaptic cleft, glutamate binds to two different types of receptors at either the postsynaptic site or on neighbouring astrocytes; ionotropic receptors and metabotropic receptors. Ionotropic receptors, including \( N \)-methyl-D-aspartate receptors (NMDARs) and 2-amino-3- (5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid receptors (AMPARs), have an ion channel pore that is gated by the extracellular binding of the glutamate agonist neurotransmitter. In contrast, metabotropic receptors are coupled with G-proteins that are activated upon glutamate binding to the receptor. Both types of receptors have been implicated in modulating synaptic strength; ionotropic through activity dependent and \( \text{Ca}^{2+} \) sensitive mechanisms, and metabotropic through the activation of second messengers required for protein synthesis (Le Duigou, Holden & Kullmann, 2011).

1.2.1 Glutamate Receptors

**NMDARs:** NMDARs are ionotropic receptors that gate non-selective cation channels upon binding of glutamate, allowing for the influx of \( \text{Ca}^{2+} \). These receptors are hetrotetrameric, comprising of two NR1 subunits that bind to the co-agonist glycine, and two NR2A, NR2B, NR2C, NR2D, NR3A, or NR3B subunits, each with unique properties that contribute to the function of the receptor. NR2A is characterized by having faster decay kinetics than other subunits, with NR1-NR2A containing receptors having a decay time constant of 118 ms compared to a time constant of 382 ms for NR1-NR2C containing receptors. NR2D subunits, however, are associated with long times courses and slow kinetics (Brothwell et al., 2008). Additionally, NMDARs are sensitive to magnesium ion (\( \text{Mg}^{2+} \)) block of current at negative potentials. This is a voltage-
dependent alteration in current-voltage relationships which NR2C subunits are less sensitive to. It is because of this Mg$^{2+}$ block of current that NMDARs act as a coincidence detector, allowing channels to be opened as long as both agonist presence and membrane depolarization occur simultaneously. A slow NMDAR-EPSC was found in the MNTB principal neuron of young (postnatal day 6-16) rats, peaking at 10 ms and decaying in an exponential manner with time constant of tens milliseconds (Forsythe & Barnes-Davies, 1993a; Barnes-Davies & Forsythe, 1995, Joshi & Wang 2002). NMDARs are strongly implicated in various forms of synaptic plasticity (reviewed in Malinow & Malenka, 2002).

**AMPA Rs:** AMPARs are non-selective cation channels upon activation. This allows for the influx of Na$^+$, efflux of K$^+$, and in some cases Ca$^{2+}$. The excitatory current generated by this receptor activates voltage-dependent channels that may contribute to action potential generation. AMPARs are heterotetrameric, and comprised of dimers of subunits GluR1, GluR2, GluR3, or GluR4, each of which have four membrane spanning segments with an extracellular N-terminus. GluR2 subunits are a dominant factor in the shape of current-voltage relationship curves and its inclusion renders the receptor impermeable to Ca$^{2+}$ (Hollmann, Hartley & Heinemann, 1991; Burnashev et al., 1992a). For receptors that are permeable to Ca$^{2+}$ (ie. receptors that do not include a GluR2 subunit) exposure to intracellular spermine inwardly rectifies current at positive potentials (Ish et al., 1995). A fast acting excitatory AMPAR-mediated excitatory postsynaptic current (AMPAR-EPSC) was found in the MNTB principal neuron of young (postnatal day 6-16) rats with a peak conductance of 37 nS, a rise time of 356 µs, and a decay time constant of 1.1 ms (Forsythe & Barnes-Davies, 1993a; Barnes-Davies & Forsythe, 1995). The fast decay observed is a result of rapid desensitization of AMPARs upon exposure to the agonist, glutamate (Verdoorn et al., 1991), an effect that can be negated
with extracellular application of cyclothiazide. At the developing calyx of Held-MNTB synapse, there is a gating switch from predominantly GluR1 to GluR4-dominant AMPARs (Joshi et al., 2004), which promotes rapid gating (Geiger et al., 1995) and is indispensable for high-fidelity neurotransmission (Yang et al., 2011).

**mGluRs:** Metabotropic glutamate receptors (mGluRs) are coupled to G-proteins. There are three major groups of mGluRs, each with their own distinct function. Group I mGluRs (mGluR1 and mGluR5) are predominantly postsynaptic and glial and have been implicated in the modulation of NMDAR activity (Skeberdis et al., 2001; Lea et al., 2002). Group II (mGluR2 and mGluR3) and group III (mGluR4, 6, 7, and mGluR8) mGluRs are more common at presynaptic sites (Shigemoto et al., 1997). In the calyx of Held, group III mGluRs are present presynaptically and suppresses VGCC current to reduce release probability (Takahashi et al., 1996; Billups et al., 2005), while postsynaptic group I mGluRs releases endocannabinoids which acts in a retrograde fashion on the presynaptic site to also negate VGCC current (Kushmerick et al., 2004). This negative feedback reduces glutamate release upon spillover of glutamate to perisynaptic sites where group 1 mGluRs are located.

### 1.3 Calyx of Held: Model of Glutamatergic Synapses

The calyx of Held is a giant glutamatergic, axosomatic synapse located in the MNTB of the brainstem, and is a critical relay in the binaural sound localization circuitry (Fig.1.1 A). The auditory brainstem network has a multitude of synapses, many of which are glutamatergic, involved in processing of acoustical signal transmission. Auditory stimuli are detected by the hair cells in the inner through vibrations of the tectorial membrane in the cochlea (Fig.1.1 A). This signal is carried by cranial nerve VIII to
spherical and globular bushy cells in the anterior ventral cochlear nucleus (aVCN) in the mammalian brainstem in a synapse called the endbulb of Held. Axons from bushy cells send two signals to the superior olivary complex (SOC); axons from the spherical bushy cells form excitatory synapses with the ipsilateral lateral superior olive (LSO), while axons from the globular bushy cells cross the midline and surround the soma of a single contralateral medial nucleus of the trapezoid body (MNTB) principal neuron, forming the excitatory calyx of Held synapse (Kuwabara, DiCaprio & Zook, 1991; Smith et al., 1991; Forsythe, 1994). The MNTB neurons in turn projects inhibitory information via a glycinergic synapse to its ipsilateral LSO, allowing for the integration of binaural auditory information (as reviewed in Tollin, 2003). The integrated brainstem auditory signal is sent through a bundle of axons via the lateral lemniscus to be further processed in the midbrain.

The calyx of Held get its name from the flower petal-like shape the presynaptic membrane forms as it wraps around the spherical MNTB principal neuron, and makes an average of 637 functional active zones (Meyer, Neher & Schneggenburger, 2001). The large presynaptic terminal that allows for electrophysiological recordings using patch-clamp techniques has made the calyx of Held a widely used model to advance current knowledge of synaptic function mechanisms. Presynaptic action potentials induce a local large brief influx Ca$^{2+}$ upon invasion of the presynaptic terminal. Glutamate loaded synaptic vesicles (SVs) in the readily releasable pool (RRP) undergo exocytosis upon high intracellular free Ca$^{2+}$ (Borst & Sakmann, 1996) and release glutamate into the synaptic cleft. (Fig.1.1 B) Excess presynaptic Ca$^{2+}$ is cleared by mitochondria in close association with SVs (Rowland, Irby & Spirou, 2000) and K$^+$ dependent Na$^+$/Ca$^{2+}$ exchangers (Kim et al., 2005). The glutamate binds to postsynaptic receptors, resulting in a large EPSC (Forsythe & Barnes-Davies, 1993b), depolarizing the membrane to cause an action potential in the MNTB principal neuron (Fig.1.1 B).
Figure 1.1 The calyx of Held is a giant glutamatergic synapse in the mammalian auditory brainstem. In the auditory brainstem circuitry (A) the calyx of Held synapse in the MNTB relays contralateral auditory information from the aVCN to the ipsilateral LSO to allow for the detection of interaural level differences for sound localization. The giant excitatory synapse releases glutamate in response to presynaptic action potentials, which bind to and open postsynaptic AMPARs and NMDARs and causes the influx of depolarising currents (B).
The MNTB relays intensity and timing information to downstream nuclei where interaural differences are computed and coded to spatially localize the source of high frequency sounds. With these interaural timing cues having differences on the sub-millisecond timescale, faithful and accurate transmission of the signals is observed in mice as young as P14 for stimuli of up to 800 Hz (Taschenberger & von Gersdorff, 2000), making the development of high synaptic fidelity a vital feature of the calyx of Held synapse.

1.4 Channels and Receptors of the Calyx of Held Synapse

The development of channels and receptors dictate many characteristics of the synapse (Table 1.1). Each structure plays a vital role in the function of the calyx of held and refines the firing properties of the synapse.

1.4.1 Presynaptic

VGCCs: The presynaptic terminal of the mouse calyx of Held initially has N-type Ca\(^{2+}\) channels, which develops to solely P/Q-type Ca\(^{2+}\) channels in the mature calyx (Forsythe et al., 1998; Iwasaki & Takahashi 1998). The P/Q-type channel demonstrates greater activity dependent facilitation, lower depression at high frequencies, and less inhibition by GABA\(_B\) than N-type (Ishikawa et al., 2005).

GABA\(_B\)R: Exogenous application of gamma-aminobutyric acid receptor B (GABA\(_B\)R) agonist revealed inhibitory GABA\(_B\)Rs suppresses presynaptic Ca\(^{2+}\) influx in a G-protein dependent manner (Takahashi Kajikawa & Tsujimoto, 1998; Yamauchi, Hori & Takahashi, 2000).

mGluRs: There has been evidence for group III mGluRs at presynaptic sites of the calyx that inhibits Ca\(^{2+}\) current and reduces probability of transmitter release.
<table>
<thead>
<tr>
<th>receptor/channel</th>
<th>location</th>
<th>subunits/type</th>
<th>function/properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPAR</td>
<td>postsynaptic</td>
<td>GluR4, GluR2 → GluR3</td>
<td>causes a large rapid EPSC; drives AP firing</td>
</tr>
<tr>
<td></td>
<td>glial</td>
<td></td>
<td>involved in synchronized firing with MNTB neuron</td>
</tr>
<tr>
<td>CBR</td>
<td>presynaptic</td>
<td>type 1</td>
<td>suppresses Ca$^{2+}$ influx</td>
</tr>
<tr>
<td>GABAR</td>
<td>presynaptic</td>
<td>B</td>
<td>suppresses Ca$^{2+}$ influx</td>
</tr>
<tr>
<td></td>
<td>postsynaptic</td>
<td>A</td>
<td>causes influx of Cl$^{-}$; inhibits MNTB AP firing</td>
</tr>
<tr>
<td>GlyR</td>
<td>postsynaptic</td>
<td></td>
<td>glycinergetic receptor; inhibits MNTB AP firing</td>
</tr>
<tr>
<td>mGluR</td>
<td>presynaptic</td>
<td>group III</td>
<td>suppresses Ca$^{2+}$ influx</td>
</tr>
<tr>
<td></td>
<td>postsynaptic</td>
<td>group I</td>
<td>release endocannabinoids for presaptic CBRs; downregulates NMDARs</td>
</tr>
<tr>
<td></td>
<td>glial</td>
<td>group II</td>
<td>involved in MNTB neuron synaptic plasticity and maturation</td>
</tr>
<tr>
<td>NMDAR</td>
<td>postsynaptic</td>
<td>NR1-1b, NR2A, NR2B → NR2C</td>
<td>causes a slow EPSC; reduced Mg$^{2+}$ block when mature</td>
</tr>
<tr>
<td>VGCC</td>
<td>presynaptic</td>
<td>N-type → P/Q-type</td>
<td>drives synaptic vesicle fusion; high facilitation, low depression, less inhibition by GABA</td>
</tr>
</tbody>
</table>
(Takahashi et al., 1996). This effect is attenuated over time such that there is less depression in mature calyces (Renden et al., 2005).

**CB1:** Endocannabinoids released by postsynaptic mGluRs bind to presynaptic type 1 cannabinoid (CB1) receptors, which negatively regulate presynaptic \( \text{Ca}^{2+} \) currents through a G-protein coupled mechanism (Kushmerick et al., 2004).

### 1.4.2 Postsynaptic and Perisynaptic

**NMDARs:** NMDARs that are found in the postsynaptic and perisynaptic sites in the MNTB principal neuron have a composition of two NR1-1b subunits, a NR2A subunit, and a NR2B subunit that declines in expression during maturation to include more NR2C subunits into the receptor triheteromeric structure (Futai et al., 2001; Steinert et al., 2010). There are few NR2D subunit-containing NMDARs in the mouse calyx, and there is not developmental change in its level of expression. There is no evidence to suggest the presence of NR3 subunits at the calyx of Held.

**AMPARs:** There is a dominance of GluR4 subunits in postsynaptic AMPARs (Barnes-Davies & Forsythe, 1995). A shift from GluR2 to GluR3 was observed during maturity (Youssoufian, Oleskevich & Walmsley, 2005), altering current-voltage relationships.

**mGluRs:** Group I mGluRs are responsible for postsynaptic release of endocannabinoids involved in retrograde inhibition of presynaptic \( \text{Ca}^{2+} \) currents (Kushmerick et al., 2004). The coincident activation of perisynaptic group I mGluRs and NMDARs due to glutamate spillover during high frequency stimulation leads to endocytosis or extrasynaptic NMDARs (Joshi, Yang & Wang, 2007).

**Inhibitory receptors:** A portion of MNTB principal neurons is sensitive to inhibitory input through GABAergic and cholinergic receptors (Wu & Kelly, 1995).
1.4.3 Astrocytes

Glial processes are associated with pre- and postsynaptic sites, forming synapse-like contacts with the calyx of Held and near active zones, actively taking up glutamate from the synaptic cleft. The neuron-glia antigen 2-positive (NG2) glia express AMPARs/kainate receptors and receive synchronized spontaneous input with the MNTB postsynaptic neuron (Muller et al., 2009; Reyes-Haro et al., 2010). Group II mGluRs were observed in glia early in postnatal development or rat calyces (Elezgarai et al., 2001) and is proposed to be involved in MNTB synaptic plasticity and maturation.

1.5 Development and Maturation of the Calyx of Held

Synaptic components are not static, but instead evolve throughout development to finally form a very specialized mature synapse. Long-term plasticity allows for several robust changes in the firing properties of the calyx of Held synapse to ensure the matured synapse relays sound-evoked electrical stimuli with high fidelity. Faithful high frequency transmission is achieved through a variety of functional and morphological changes in the calyx (Fig.1.2). In mice, the large excitatory synapse forms at postnatal day 3 (P3). The calyx terminal matures at P5 into a spoon-shaped structure with multiple cytoplasmic projections around the MNTB principal neuron (Kandler & Friauf, 1993). The immature presynaptic terminal expresses a combination of N-type and P/Q-type VGCCs (P4 - P9). Cytoplasmic projections start to disappear at P8, and N-type VGCCs diminish after P7, disappearing from the presynaptic terminal by P10 (Iwasaki & Takahashi, 1998). P/Q-type channels are responsible for facilitation, depression recovery due to insensitivity to GABA-induced depression, and are more tightly coupled to vesicles than N-type (Inchauspe, Forsythe & Uchitel, 2007; Kochubey, Han & Schneggenburger, 2009), making the presynaptic terminal highly efficient at SV release.
There is a steady increase in amplitudes of NMDAR-EPSCs in immature (P7 - P12) MNTB neurons, peaking in amplitude at approximately P11 (Joshi & Wang, 2002). At around P12, the ear canals of mice open and the mouse receives external auditory stimulation for the first time. The opening of the ear canals coincides with a rapid downregulation of NMDARs (Blatchley, Cooper & Coleman, 1987; Geal-Dor et al., 1993). By reducing the slow NMDAR component of the postsynaptic response, recovery of the membrane from prior depolarisations is more rapid, increasing the likelihood of voltage gated channels responding to subsequent depolarisations.

In parallel with the NMDAR reduction, a different population of AMPARs, comprising of GluR3/4 subunits instead of GluR2, are upregulated in the MNTB neurons after the onset of hearing. In the immature calyx of Held synapse, AMPAR-EPSCs are short with slow decay kinetics brought about by faster deactivation of receptors (Koike-Tani, Saitoh & Takahashi, 2005). AMPAR-EPSCs in the mature calyx (P14) are larger in amplitude and display faster desensitisation kinetics (Ishikawa & Takahashi, 2001; Joshi & Wang, 2002; Youssoufian, Oleskevich & Walmsley, 2005). Also at P14, full adult morphology of the synapse is achieved, existing as a multi-digit structure around the postsynaptic neuron believed to enhance rapid diffusion of glutamate out of the synaptic cleft and reduce AMPAR desensitization. This fenestration occurs with age regardless of auditory stimuli (Youssoufian, Couchman & Walmsley, 2008). The number of SVs in the readily releasable pool (RRP) of the matured presynaptic terminal doubles compared to immature synapses, while the release probability for each vesicle halves (Iwasaki & Takahashi, 2001). These changes serve to provide a rapid and robust response to synaptic stimuli with swift decay kinetics to allow for faithful synaptic firing during high frequency stimulation.
Figure 1.2 *The calyx of Held – MNTB principal neuron synapse undergoes several changes during its two weeks of development.* NMDAR-EPSCs increase steadily after the formation of the synapse and peaks at P10. At around P12 the ear canals of the mouse open and receive auditory stimuli for the first time. After this, NMDARs are rapidly downregulated. This coincides with a subunit switch from NR2B to NR2C containing NMDARs, which alters the current-voltage relationship. AMPAR-EPSCs increase in amplitude during development, drastically so after the onset of hearing. This upregulation is accompanied by a subunit switch to more rapidly gated GluR3/4 containing AMPARs, resulting in a short and wide AMPAR-EPSC in the immature neuron which develops to a large and sharp AMPAR-EPSC. In addition, dramatic morphological changes occur in the presynaptic space. The immature calyx is spoon-shaped and wraps around the MNTB principal neuron. This matures into a more digitated structure with multiple fenestrations and swellings, a change that is believed to promote glutamate clearance and therefore aid in synaptic fidelity during high frequency trains of stimuli.
1.6 Short-Term Plasticity in the Calyx of Held

The calyx of Held synapse has several specializations that allow it to experience various forms of short-term plasticity. In response to a presynaptic action potential, only 20% of the multitude of synaptic vesicles in the readily releasable pool (RRP) fuses due to low release probability (Schneggenburger, Meyer & Neher, 1999; Yang et al., 2009) causing a non-saturation of postsynaptic receptors (Schneggenburger & Neher, 2000). This allows for sensitivity to modulating synaptic strength and for enabling multiple EPSCs during high frequency stimulation.

As the calyx of Held relays high frequency auditory signals, the postsynaptic MNTB principal neuron must be able to detect and fire action potentials in response to rapid repeated stimulation. Stimulating the presynaptic axon with a 1 Hz train can reduce postsynaptic currents in the principal neuron of the medial nucleus of the trapezoid body (MNTB) to half the initial value, and to 10% the initial value with 100 Hz trains. This form of short-term depression is induced by a combination of reduced neurotransmitter release due to presynaptic SV depletion (von Gersdorff et al., 1997) and desensitisation of postsynaptic AMPARs (Wong et al., 2003). Desensitisation of the postsynaptic receptors in the calyx of Held synapse also contributes to the decrease of spontaneous current amplitudes after prolonged synaptic stimulation in immature synapses but become negligible in mature synapses (Neher & Sakaba, 2001, Joshi & Wang 2002; Yang et al., 2011).

Studying the properties of EPSCs during a pair of stimulating pulses reveals several details about the presynaptic machinery in a synapse. Paired-pulse facilitation of 20% is observed with interpulse intervals of 5-10 ms due to Ca$^{2+}$ build-up from the initial pulse that prolongs the release of neurotransmitter (Cuttle et al., 1998; Chen & Regehr, 1999). In addition, paired-pulse facilitation can be partially accounted for by residual Ca$^{2+}$
from the previous stimuli, and its activation of neuronal Ca$^{2+}$ sensor 1, which may facilitate further presynaptic Ca$^{2+}$ currents and its efficiency (Tsujimoto et al., 2002; Müller, Felmy & Schneggenburger, 2008). Paired-pulse depression is common in the calyx of Held before the onset of hearing, significantly contributed by AMPAR desensitisation from previous glutamate exposure (Koike-Tani et al., 2008). Paired-pulse depression can also provide insights into the probability of release for synaptic vesicles. A large probability of release will cause the fusion of more SVs, further depleting the RRP and causing much fewer SV fusion events during the subsequent stimulation.

Previous studies have shown that synaptic depression is largely accounted for by a depletion of synaptic vesicles in the readily releasable pool (RRP) and AMPAR desensitization in immature synapses. Recovery from synaptic depression reflects primarily the replenishment of RRP. Interestingly the recovery is frequency-dependent, with the speed of recovery increasing with higher stimulation frequencies due to Ca$^{2+}$/calmodulin-dependent replenishment (Wang & Kaczmarek, 1998; Sakaba & Neher, 2001). At higher frequencies, the recovery is further divided into a fast and slow component, with the fast recovery component dependent on presynaptic Ca$^{2+}$, leading to the conclusion that Ca$^{2+}$ influx through presynaptic APs is a key signal that regulates the replenishment of the RRP of synaptic vesicles in the presynaptic terminal of the calyx of Held.

### 1.7 NMDAR-Dependent Synaptic Plasticity: A Hypothesis

NMDA receptors have long been suggested to be critical for development, playing a key role in several processes that regulate and refine synaptic responses. Research indicates NMDARs are involved in various forms of synaptic plasticity, including long-term potentiation and long-term depression during high frequency
stimulation (Harris, Ganong & Cotman, 1984; Herron, et al. 1986). NMDARs also play a role in the refinement and consolidation of synapses in the solitary nucleus (Vincent et al., 2004) and trigeminal principal nucleus (Lee, Lo & Erzurumlu, 2005) in the rodent brainstem. Adding to its significant contributions in synaptic maturation, the vital receptor is responsible for morphological development of synapses through the patterning of presynaptic and postsynaptic elements, as previously observed in hippocampal spines (Merriam et al., 2011). Activation of postsynaptic NMDARs has also been shown to send retrograde signals to the presynaptic terminal, which can regulate the stability of presynaptic contacts and axon arbor structure (Ramoa et al., 2001; Ruthazer, Akerman & Cline, 2003), while NMDAR-mediated synaptic activity in adult mammals promotes the integration of newly born neurons into the mature circuit (Tashiro et al., 2006), activate local protein synthesis (Huang et al., 2002), and promote the insertion of AMPARs into the postsynaptic densities (reviewed in Malinow & Malenka, 2002).

Considering the vast role NMDARs play in development, we were intrigued by the previously observed high levels of functional NMDARs in the calyx of Held which peaks during the few days prior to the onset of hearing (Joshi & Wang, 2002). Several developmental changes occur in the short time period just before and after the onset of hearing that primes the synapse to relay high frequency transmission. The addition of fast-gating GluR4 subtype AMPARs in the calyx is beneficial in achieving high synaptic fidelity upon the onset of hearing, while the expression of NMDARs hinders faithful firing at high frequencies due to its slow kinetics. Therefore NMDARs must provide the calyx of Held synapse with a vital feature to warrant its initial upregulation. We thereby hypothesize that NMDARs are indispensable for the functional and morphological maturation of the calyx of Held-MNTB principal neuron synapse and dictates the properties of the adult synapse that allows for high fidelity of synaptic and consequently acoustic neurotransmission.
In order to investigate the purpose of abundant NMDARs in pre-hearing mouse MNTB neurons, we sought to study the effects of drastically attenuating NMDARs on developmental plasticity at the calyx of Held-MNTB synapse. However, genetically eliminating NMDARs in mice results in complete offspring fatality due to respiratory failure and impairment of the suckling response (Forrest et al., 1994; Kutsuwada et al., 1996). We therefore utilized a genetic knockdown of the NR1-subunit of NMDARs, which globally reduces functional receptors in mice. Using this tool, we studied the impact NMDARs have on functional and morphological remodeling at the calyx of Held during the critical window of maturation between P9 and P18.

1.8 NR1-KD: Genetic Model of NMDAR Hypofunction

We attenuated NMDAR function through the use of the global knockdown of the NR1 subunit (NR1-KD) in mice, as developed by the Koller laboratory (Mohn et al. 1999). The genetic mutation involved in the NR1-KD is an approximately 2 kb foreign DNA insertion into an intron near the 3’ end of the Grin1 allele (Fig.1.3 A-B). The targeted insertion of this DNA increases the amount of truncated mRNA, most likely due to premature termination of transcription at the insertion site. This reduces the level of functional NMDARs to an estimated 10-20% of normal levels, allowing for sufficient NR1 subunits to support survival into adulthood. This survival may be due to the only 60% reduction of functional NMDARs, as measured through radioligand binding with [3H]MK-801 (reviewed in Ramsey, 2009; Fig.1.3 C), which is possibly why NR1-KD mice are capable of suckling and breathing, unlike NR1-null mice.

Since its development, the NR1-KD has been used as a genetic model to study the role of NMDARs, especially in schizophrenic pathophysiology. Behaviourally, NR1-KD mice are consistently socially withdrawn and fail to habituate to novel environments.
Figure 1.3 Amy Ramsey’s genetic model of NMDAR hypofunction utilizes a NR1-KD (figure from Ramsey, 2009). An insertion of neo DNA into the Grin1 allele leads to a hypomorphic mutation where there is a limited number of functional NR1 subunits produced (B) in the NR1-KD after transcription and splicing as opposed to the excess produced in the WT (A). This reduces the total number of functional NMDARs produced in the NR1-KD mouse. Functional NMDAR levels (C), as measured through radioligand binding of antagonist [3H]MK-801, are reduced throughout the brain. Darker shades represent higher levels of NMDAR function. (reprinted with the permission of the author)

(Mohn, et al., 1999; Duncan et al., 2004, 2009; Halene et al., 2009). They have an exaggerated startle response to auditory stimulus (Duncan et al., 2006) though a deficit in sensory processing and selective attention, as measured through the pre-pulse inhibition paradigm of acoustic startle responses (Bickel, Lipp & Umbricht, 2006, 2008; Halene et al., 2009). In spite of its creation more than a decade ago, there is few, if any, functional assays done at the synaptic level for basal neurotransmission and acute long-term plasticity in the NR1-KD, nor are there any cross-correlation studies on
morphological and electrophysiological analyses. Using the mouse calyx of Held-MNTB synapse as a model, we set forth to go beyond the behavioural studies and investigate how development of excitatory glutamatergic synapses are affected by significantly reducing the level of functional NMDA receptors.

1.9 Summary of Working Hypothesis and Objectives

Several changes occur both functionally and morphologically in the calyx of Held-MNTB principal neuron synapse during development that promote high frequency neurotransmission and high synaptic fidelity. However the peak in NMDAR-EPSCs just prior to the onset of hearing hinders faithful transmission due to the receptor’s slow gating kinetics. Yet, high NMDAR activity as well as the specific NR2B-to-NR2C subunit switch is observed in several other developing glutamatergic synapses. Therefore, NMDARs are classically hypothesized to be vital in driving the developmental process either through their function or their physical presence. We tested this hypothesis using the NR1-KD to model NMDAR hypofunction and evaluated the development of the calyx of Held-MNTB principal neuron synapse at multiple levels: synaptic function, presynaptic morphology, and auditory transmission at the brainstem.
CHAPTER TWO: Experimental Protocol

2.1 Animals

A hypomorphic Grin1 allele of the NR1 subunit of NMDARs developed by the Koller lab in the University of North Carolina (Mohn, et al., 1999) is used to attenuate NMDAR expression in the whole mouse by reducing the transcription of the essential NMDAR subunit, NR1. The NR1 knockdowns (NR1-KD) have a total expression of 5% - 10% of the normal abundance, significantly reducing NMDAR expression while allowing survival into adulthood, unlike NR1 knockouts which die 8-15 hours after birth (Forrest, et al., 1994; Li et al., 1994). Heterozygous breeding pairs of 129X1SV/J: Grin1tmunc1 +/- males and C57BL/6J: Grin1tmunc1 +/- females were used to generate offspring for all experiments. All studies involving the NR1-KD utilized their wild-type littermates (WT) as control.

2.2 Electrophysiology

2.2.1 Slice Preparation

Mouse pups aged between P9 to P18 were decapitated using a small guillotine. 250 µm thick transverse slices of the auditory brainstem were cut using a Leica VT1000S Microtome (Leica Microsystems). During the dissection, the brain was submerged in an ice cold artificial cerebral spinal fluid (aCSF) (pH7.3) containing (in mM): NaCl (125.0), KCl (2.5), glucose (10.0), NaH2PO4 (1.25), myo-inositol (3.0), Na pyruvate (2.0), L-ascorbic acid (0.5), NaHCO3 (26.0), CaCl2 (0.1), and MgCl2 (3.0), oxygenated with 95% O2 and 5% CO2 (Table 2.1) The slices were subsequently incubated for an hour in an oxygenated 37°C incubation aCSF solution similar to the
above dissection solution except with (in mM) CaCl$_2$ (2.0) and MgCl$_2$ (1.0).

Whole cell patch clamp recordings were performed on the MNTB principal neuron at room temperature (~20-24°C). Slices were perfused with an oxygenated extracellular solution containing the incubation aCSF with added 0.1 nM glycine, a co-agonist of NMDA receptors, and 0.001 mM strychnine and 0.01 mM bicuculine to block inhibitory glycinergic and GABAergic inputs, respectively (Table 2.1). A bipolar platinum stimulation electrode was placed near the midline, along the axons of globular bushy cells presynaptic to the MNTB (Fig.2.1). Recording microelectrodes were pulled from glass borosilicate to a resistance of 2.5 MΩ - 3.0 MΩ and, in order to solely measure EPSCs, were loaded with an intracellular solution containing (in mM): K-gluconate (97.5), CsCl (32.5), ethylene glycol tetraacetic acid (EGTA) (5.0), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10.0), MgCl$_2$ (1.0), sodium channel blocker QX-314 bromide (3.0), potassium channel blocker tetraethylammonium (TEA) (30.0), and spermine (0.2), an inward rectifier of Ca$^{2+}$ permeable AMPARs (Table 2.2). Adding spermine to the intracellular solution prevents the gradual diffusion and washout of endogenous spermine present in the MNTB principal neuron. Using a SIU5 S880 stimulator (GRASS Instrument Division; Astro Med Inc.), slices were stimulated at 120% of the stimulation required to faithfully evoke EPSCs. Recordings were performed using a MultiClamp 700B amplifier and digitized by Digidata 1440A (Axon Instruments, Molecular Devices), series resistance was maintained at 3.0-4.5 mΩ and compensation with of 60% prediction and 90% correction.

Through analysis via Clampfit10 (Axon Instruments, Molecular Devices) MiniAnalysis (Synaptosoft) software, developmental synaptic profiles were created over five age groups (P9/P10, P11/P12, P13/P14, P15/P16, and P17/P18) of the NR1-KD calyx of Held and compared to their respective WT littermate.
Figure 2.1 Electrophysiological recordings were made from the MNTB principal neuron in brainstem slices. A stimulation electrode placed near the midline of the brainstem slice allows us to stimulate the presynaptic axons of the calyx of Held synapse. We can record detect and analyse EPSCs through whole-cell patch clamp recordings of the MNTB principal neuron.
Table 2.1 Extracellular solution composition

<table>
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<tr>
<th>solution</th>
<th>compound</th>
<th>amount (mM)</th>
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<tbody>
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<td>basic aCSF</td>
<td>sodium chloride</td>
<td>NaCl 125.0</td>
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<td></td>
<td>potassium chloride</td>
<td>KCl 2.5</td>
</tr>
<tr>
<td></td>
<td>glucose</td>
<td>C₆H₁₂O₆ 10.0</td>
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<tr>
<td></td>
<td>sodium dihydrogen phosphate</td>
<td>NaH₂PO₄ 1.25</td>
</tr>
<tr>
<td></td>
<td>sodium pyruvate</td>
<td>Na⁺ C₃H₃O₃⁻ 2.0</td>
</tr>
<tr>
<td></td>
<td>myo-inositol</td>
<td>C₆H₁₂O₆ 3.0</td>
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<td></td>
<td>L-ascorbic acid</td>
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<td>sodium bicarbonate</td>
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<td>added to dissection</td>
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<tr>
<td></td>
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<td>MgCl₂ 1.0</td>
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<td>bicuculline</td>
<td>C₂₀H₁₇NO₆ 0.01 *GABA&lt;sub&gt;A&lt;/sub&gt; antagonist</td>
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<td>strychnine</td>
<td>C₂₁H₂₂N₂O₂ 0.001 *glycinergic antagonist</td>
</tr>
<tr>
<td></td>
<td>glycine</td>
<td>C₂H₅NO₂ 0.00001 *NMDAR co-agonist</td>
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</tbody>
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*partly or **fully dissociates to contribute to ionic composition
### Table 2.2 Intracellular solution composition

<table>
<thead>
<tr>
<th>solution</th>
<th>compound</th>
<th>amount (mM)</th>
<th>note</th>
</tr>
</thead>
</table>
| **V-clamp recordings** | potassium gluconate     | K⁺ C₆H₁₁O₇⁻ | 97.5  
|                     | cesium chloride         | CsCl        | 32.5  
|                     | EGTA                    | C₁₄H₂₄N₂O₁₀ | 5.0 *free Ca²⁺ buffer    
|                     | HEPES                   | C₈H₁₈N₂O₄S | 10.0 *pH buffer              |
|                     | magnesium chloride      | MgCl₂       | 1.0  
|                     | QX-314 bromide          | Br⁻ C₁₆H₂₇N₂O⁺ | 3.0 *blocks voltage-activated Na⁺ channel current |
|                     | TEA                     | C₈H₂₀N⁺    | 30.0 *blocks K⁺ channels      |
|                     | spermine                | C₁₀H₂₆N₄  | 0.2 *prevents endogenous spermine washout; *inwardly rectifies AMPAR current at positive Vₘ |

| **I-clamp recordings** | potassium gluconate     | K⁺ C₆H₁₁O₇⁻ | 97.5  
|                       | potassium chloride      | KCl         | 32.5  
|                       | EGTA                    | C₁₄H₂₄N₂O₁₀ | 5.0 *free Ca²⁺ buffer    
|                       | HEPES                   | C₈H₁₈N₂O₄S | 10.0 *pH buffer              |
|                       | magnesium chloride      | MgCl₂       | 1.0  
|                       | NMDG                    | C₇H₁₇NO₅  | 40.0 *pH buffer             |

* blacklist.
2.2.2 AMPAR and NMDAR Developmental Profiles

Using whole-cell configuration, NMDAR-EPSCs were isolated by holding the cell at +60 mV during stimulation. At this holding potential spermine, effectively blocks Ca\(^{2+}\) permeable AMPAR currents. Synaptic NMDAR-EPSCs were measured by delivering a single stimulating pulse at the positive holding potential, activating receptors localized near active zones at the post-synaptic densities (Fig.3.1 A). A combination of synaptic and extrasynaptic NMDAR-EPSCs was measured using a 100 Hz 100 ms stimulation train that causes glutamate to spill over to perisynaptic sites. Amplitude was measured as the plateau current during the 100 Hz train (Fig.3.1 C). AMPAR-EPSCs, on the other hand, were isolated by maintaining the cell’s holding potential (\(V_h\)) at –60 mV during stimulation (Fig.3.2 A). At this holding potential, NMDAR-EPSCs are blocked by Mg\(^{2+}\). AMPAR-EPSC waveform properties of the WT and NR1-KD calyx of Held synapse were analysed during development (P9-P18), including amplitude, 10-90% rise time, and the fast and slow decay time constant (\(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\)). In addition, synaptic AMPAR and NMDAR amplitude current-voltage relationships were measured between -60 mV and +60 mV at every 20 mV interval (S1 and S2). From this, we measured the comparative inward rectification of current at positive potentials as an indirect method to compare the GluR2 expression in AMPARs. We performed this evaluation by studying the relative AMPAR-EPSC amplitudes at a \(V_h\) of –40 mV (\(I_{-40}\)) and +40 mV (\(I_{+40}\)).

Additionally, we measured the properties of quantal events by recording spontaneous release via miniature EPSCs (mEPSCs) at –60 mV holding potential for 3 minutes without compensation (Fig.3.3 A). Amplitude, decay and 10-90% rise time were analysed. Frequency of mEPSCs was compared by measuring inter-event intervals and compiled into cumulative histogram for each group (age and genotype).
2.2.3 Short-Term Depression and Recovery

During maturation, the calyx of Held-MNTB principal neuron synapse undergoes drastic alterations in short-term depression (STD). We evaluated the development of STD and recovery properties of the NR1-KD and WT synapses through the use of high frequency trains, which simulates the synaptic fidelity needed to faithfully transmit sound stimuli. In the whole-cell configuration, under voltage-clamped compensated conditions at –60 mV, we stimulated the MNTB neuron at 120% the stimulation threshold for 100 ms at 50 Hz, 100 Hz, and every 100 Hz increment until there were multiple failures to induce EPSCs (Fig.3.4 and Fig.3.5). Amplitudes of each EPSC in the train was measured and expressed as a percentage of the amplitude of the first EPSC in the train. As a measure of release probabilities for each group, paired pulse ratios (PPRs) were calculated using the same inter-event intervals associated with the above frequencies. PPRs were measured as the relative amplitude of an AMPAR-EPSC \( A_2 \) as a proportion of the amplitude of an initial AMPAR-EPSC \( A_1 \) that occurred 3.3 ms, 5.0 ms, and 10 ms prior (Fig.3.6 A-B). The amplitudes of the last five AMPAR-EPSCs in 100 ms 300 Hz trains were averaged for each cell to measure the steady state of SV replenishment for each age group apart from P9/10, where we were unable to obtain reliable firing at that frequency (Fig.3.6 C).

We measured the rate of synaptic vesicle (SV) recovery after depleting the readily releasable pool (RRP) with a 300 Hz 100 ms train. We waited various time intervals from the end of the first train (every 80 ms between 20 ms and 900 ms, and every 1 second up to a 13 second interval) to partially replenish the RRP before stimulating with a second identical train to deplete the RRP once again (Fig.3.7 A). Each pair of trains was separated by at least 30 seconds of inactivity to ensure full recovery of SVs before the next interval was recorded. The mean amplitude of the last five EPSCs in the second train represented the steady state of replenishment and was subtracted from
each EPSC amplitude in both trains. The percentage of recovery at each interval was calculated as the summation of currents of the second train expressed as a percentage of the summated currents of the initial train. The percentages were plotted against the duration of the inter-train interval and fit with a cumulative probability double exponential function to attain a slow and fast time constant.

2.2.4 Excitability

Excitability studies were performed only in the P17/18 age group. In these sets of experiments, the extracellular solution did not include glycine and the recording microelectrode contained a K+-based intracellular solution containing (in mM): K-gluconate (97.5), KCl (32.5), EGTA (5.0), HEPES (10.0), MgCl$_2$ (1.0), and N-methyl-d-glucamine, NMDG buffer (40.0) (Table 2.2). Prior to breaking through the membrane of the MNTB principal neuron, we measured changes in local membrane current in cell-attached mode holding at –60 mV. At twice the threshold to elicit a response, we stimulated with frequencies of 100 Hz, 200 Hz, 300 Hz, and 400 Hz for 400 ms duration. This was repeated after breaking through the membrane. In both cases the number of failures to induce a response were recorded for each frequency (Fig.3.9).

Switching to a current-clamp configuration, current was injected into the MNTB principal neuron to achieve a resting potential of –70 mV, if needed. We repeated the voltage-clamp stimulation protocol of 400 ms trains of 100 Hz, 200 Hz, 300 Hz, and 400 Hz and recorded the postsynaptic APs generated. Once again, the number and percentage of failures were measured. In addition, 200 ms current steps of –0.1 nA to 1.8 nA at every 0.1 pA interval were injected into the MNTB principal neuron every 30 seconds to measure excitability (Fig.3.8). From this we evaluated changes in membrane potential ($V_m$) and measured AP spiking in response to the current injections in the MNTB principal neuron.
2.3 Presynaptic Morphology Imaging

Morphology of the presynaptic terminals of immature (P11) and mature (P17) calyces was visualized through anterograde tracing by 1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil; Molecular Probes, Invitrogen) crystals, a lipophilic membrane stain which fluoresces in the 543 – 735 nm range. Mice were decapitated and brains isolated as detailed in section 2.2. The block of brain was then fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Dil crystals were embedded into the cochlear nucleus of the fixed brain and incubated for 4 – 6 weeks at 37°C to fully stain the entire cell, reaching the presynaptic axons and laterally diffusing into the presynaptic membrane of the calyx of Held. The brain was then submerged in a phosphate buffered saline (PBS) and sliced to a thickness of 200 – 250 µm (Fig.3.10). Three-dimensional images of the calyceal structures were compiled through a z-stack reconstruction of high resolution confocal images at 0.81 µm depth intervals acquired with a Carl Zeiss LSM 510 laser scanning microscope fitted with a 543 nm laser.

Images were analyzed using Volocity 3-D Image Analysis Software (PerkinElmer). Objects were selected from fully dyed presynaptic calyces, excluding the axon. Fluorescence intensities lower than 4% were excluded from the measurements to omit the larger than actual perimeter due to diffusion of the fluorescence. Fluorescent noise was removed with a fine filter and objects less than 1.5 µm in volume were excluded. From this we determined the volume and surface area of the presynaptic space and diameter of the MNTB principal neuron. In addition the number of swellings were counted to determine the class of calyces as detailed by Grande and Wang (2011). Though the classification system was only previously applied to mature calyces, we have used the same parameters to study the immature (P11) calyces. We further
counted the number of visible or partially visible presynaptic terminals in set field windows at varying depths of the MNTB to determine the density of calyces present in each group.

2.4 Auditory Brainstem Responses (ABR)

ABRs record electrical activities associated with various relays of the auditory brainstem, and were used to measure the success of acoustical sound detection and transmission in WT and NR1-KD mice. After the onset of hearing, NR1-KD and WT mice aged P15-P18 were anesthetized with a 20 mg/kg xylazine and 100 mg/kg ketamine cocktail in a sterile saline solution via an intraperitoneal injection. This sedation and paralysis eliminated movement-induce noise in the ABR recordings. In a sound attenuating room, recording electrodes were placed subcutaneously behind each pinna and a reference electrode under the skin along the midline at the back of the head. Sound stimuli were introduced monaurally via a transducer (Intelligent Hearing Systems) attached to a connecting tube with a probe tip inserted into the ear canal of the mouse pup.

In vivo auditory brainstem potentials were recorded and digitized for a duration of 50 ms before and after repeated 100 ms clicks of auditory stimuli. The clicks were delivered at several levels of intensity between 0 dB and 80 dB at 10 dB intervals. An average of 300 recordings that passed through an 11% noise filter were compiled using the SmartEP platform (Intelligent Hearing Systems software) (Fig.3.11 A). Numerical data acquired from the recordings were transferred to a MS Office Excel worksheet to set appropriate display parameters, converted to a text file, and analyzed using Clampfit10 (Axon Instruments) software. Hearing threshold for each ear was taken as the last dB recording in which the five ABR waves could be identified. Peak amplitude
from the base of the previous wave (or baseline for the initial wave) was measured, as 
was peak latency and the descending slope of each wave. Values were averaged for 
each dB level for the NR1-KD and WT mouse pups.

2.5 Statistics

Unless otherwise specified, all data were averaged for each cell. The statistics 
for each group is expressed as mean ±SEM with significance between NR1 and WT 
data measured using unpaired, two-tailed student t-tests. P-values, the probability of the 
null hypothesis, are indicated in figures as p<0.05 (*), p<0.01 (**), and p<0.001 (***)
Developmental profiles were compiled over five age groups (P9/P10, P11/P12, P13/P14, 
P15/P16, and P17/P18) and the properties of the NR1-KD properties were compared to 
WT properties within the respective age groups.

The decay time course of the average AMPAR-EPSC for each neuron was fit 
with a standard double exponential curve to determine the two τ components, while 
NMDAR-EPSCs were fit with a single exponential function.

The frequencies of spontaneous mEPSCs in each age group were compared by 
measuring the inter-event interval and compiling the data for each group. Expressing the 
data in a cumulative histogram allows for the NR1-KD and WT data to be compared 
using a Kolmogorov-Smirnov (KS) test to determine the p-value. The decay of each 
spontaneous mEPSCs was fit with a single exponential curve. Data was for each age 
group was compiled and the τ values were expressed in a conventional histogram to 
determine the distribution of the time constants.

STD during trains were plotted against time, averaged for the each group, and 
fitted with a single exponential decay. Recovery of EPSCs were also plotted against the 
interval between in the last EPSC of the first train and the first EPSC of the second train,
but fit with a cumulative probability double exponential curve.

In response to current injections, the membrane potential of the MNTB principal neuron changed. The change was plotted against the injected current. The average of several trials was fit with a single exponential cumulative probability curve. The number of APs fired during the step injections were also plotted against the current and averaged over several trials. The data was then fit with a Boltzmann charge-voltage curve with the following equation:

\[
f_t(V) = \frac{I_{\text{max}} a x}{1 + e^{(V_{\text{m}/d} - V)/\nu_c}} + C
\]

In studying the morphology of the calyx of Held, diameter, volume and surface area were averaged. In addition, we also expressed volume and surface are in conventional histograms to evaluate the comparative distribution of the measurements between the WT and NR1-KD data.
CHAPTER THREE:
Results

3.1 Robust reduction of synaptic NMDARs at the MNTB principal neuron in the NR1-KD

It was previously determined expression of functional NMDARs in the brainstem, as determined through radioligand binding of antagonist [3H]MK-801, is reduced by 60% in the NR1-KD mouse (Mohn et al., 1999; reviewed in Ramsey, 2009). However, it is still unknown how much NMDARs are decreased specifically in the MNTB principal neuron. This is further complicated by the varying levels of NMDARs during the first few weeks of development (Joshi & Wang, 2002). For this reason, we first wanted to determine if NMDARs are in fact lowered in the MNTB principal neuron of the NR1-KD mouse, and, if so, by how much at different ages. The expression of synaptic NMDA receptors was measured as the amplitude of NMDAR-EPSCs after delivering a single stimulating pulse at +60 mV holding potential (V_h) (Fig. 3.1 A-B). At the age group P9/10, single NMDAR-EPSCs in NR1-KD calyx of Held synapses were significantly smaller by 71.5% (p=0.0145), with NMDAR-EPSC amplitudes of WT neurons 3.615 nA ± 0.760 nA (mean ± SEM) and NR1-KD neurons 1.026 nA ± 0.258 nA. A similar lower amplitude by 65.8% (p=0.007) was also observed in single NMDAR-EPSCs in the P11/12 age group, coinciding with the peak of NMDAR amplitudes in both the WT at 4.152 nA ± 0.389 nA as well as the NR1-KD at 1.419 nA ± 0.377 nA. At approximately P12, the ear canals open and the mouse receives auditory stimuli. In the following days there is a dramatic decrease of NMDARs in both the WT and NR1-KD MNTB principal neurons, as previously described in Joshi and Wang (2002). There were no significant differences in synaptic NMDAR-EPSCs between WT and NR1-KD calyx of Held synapses through
Figure 3.1 **NMDAR-EPSC development profiles reveal a large decrease in current amplitude of the NR1-KD synapse prior to the onset of hearing.** Stimulating with a single pulse while holding the MNTB principal neuron at +60 mV provides us with the current of synaptic NMDARs (example trace A), while delivering a train of stimuli at 100 Hz 100 ms causes a summation of synaptic and extrasynaptic NMDAR-EPSCs (example trace C). Developmental profiles of these currents reveal a significant decrease in current amplitudes of both synaptic (B) and summated (D) current, especially in the pre-hearing onset (<P12) mice. This was accompanied by slower decay of synaptic currents (E). Stimulating at several holding potentials (F) show there is no alteration in the current-voltage relationships (P9/10 shown; G), mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 3, 5, 4, 4, 5 neurons for WT, respectively, and n = 4, 6, 6, 5, 10 neurons for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001.

ages P13-P16. However, at P17/18 a difference was observed again with a mean NMDAR-EPSC amplitude of 0.674 nA ±0.059 nA for WT and 57.8% lower 0.273 nA ±0.054 nA for the NR1-KD (p=0.0009). Apart from an overall decrease in amplitude, no differences were observed in the current-voltage relationships of NMDARs between WT and NR1-KD in all age groups (Fig.3.1 F-G, S1).

Because a single stimulation does not saturate postsynaptic NMDARs (Wang, 2000), we measured cumulative NMDAR-EPSCs by stimulating the presynaptic axon with a 100 Hz 100 ms train at +60 mV V_h. A train of stimuli loads the synaptic cleft with glutamate and also spills glutamate onto extrasynaptic sites, activating NMDARs and causing an summated response in the NMDAR-EPSC amplitude after each stimulating event (Fig.3.1 C-D). Summated NMDAR-EPSC amplitudes in the NR1-KD neuron at P9/10, though not significantly so, was 53.2% lower (p=0.0709) at 3.102 nA ±0.787 nA compared to 6.626 nA ±1.466 nA in the WT neuron. Reduced summated NMDAR-EPSC amplitudes were also observed in P11/12 with means of 2.999 nA ±0.575 nA in NR1-KD MNTB neurons also 53.8% lower than WT neurons at 6.501 nA ±0.580 nA (p=0.0021). Following the same profile as synaptic NMDARs, there is no significant difference between WT and NR1-KD summated NMDAR-EPSC amplitudes during ages P13-16. AT P17/18 amplitudes of summated NMDAR-EPSCs the NR1-KD MNTB neuron are
once again 46.9% lower (p=0.0253) than WT neurons at 0.818 nA ±0.129 nA compared to 1.543 nA ±0.320 nA. At every age group, the percentage of decrease in single NMDAR-EPSCs from the NR1-KD synapses is greater than summated NMDAR-EPSCs reflecting combined activity of synaptic and extrasynaptic NMDARs, suggesting that not only a reduced synaptic NMDARs, but also potentially a selective redistribution of NMDARs away from synaptic sites into more perisynaptic areas of the NR1-KD MNTB principal neuron. Hence, NR1 subunit may be important for targeting NMDARs to the synaptic domain.

As the auditory brainstem matures, single NMDAR-EPSCs display faster decay kinetics at the calyx of Held synapse (Joshi & Wang, 2002). Being interested in if attenuated NMDAR level in the NR1-KD affects kinetics of NMDAR-EPSCs, we fit the decay time course of NMDAR-EPSCs with a single exponential function (Fig.3.1 E). The fast time constant in the WT synapse steadily decreases from 60.29 ms ±3.78 ms at P9/10 to 26.61 ms ±2.66 ms at P17/18. Through all age groups, synaptic NMDAR-EPSCs in the NR1-KD calyx of Held synapse have a much slower decay with a fast time constant of 118.18 ms ±15.17 ms at P9/10, 96% longer than WT (p=0.0208), and also decreases during maturation to 44.54 ms ±2.03 ms by P17/18, 67.4% slower than WT (p=0.0002). These consistently slower decay time constants may be associated with a change in the subunit composition of synaptic NMDARs (e.g. slow NR2B vs fast NR2A), but the fact that the NR1-KD MNTB neuron has a larger distribution of NMDARs away from synaptic active zones as reflected by summated NMDAR-EPSCs, an alternative interpretation is that glutamate isn’t cleared from all receptors as swiftly as the neurotransmitter spreads along the synaptic cleft. As a result, NMDARs remain activated for a longer period of time, resulting in slower decay kinetics in the NR1-KD calyx of Held.
3.2 NR1-KDs have altered AMPAR-EPSCs in the MNTB principal neuron differentially during development

To determine whether this dramatic reduction of NMDARs in the MNTB principal neuron affects the development of functional synaptic AMPARs, we analysed evoked AMPAR-EPSCs over various age groups (Fig.3.2 A-C). This was achieved by delivering a single stimulating pulse to the presynaptic axon while holding the MNTB principal neuron at –60 mV. The AMPAR-EPSC kinetics of both, the WT and NR1-KD, calyx of Held synapse follow previously observed maturation to larger and sharper waveforms (Joshi & Wang, 2002; Iwasaki & Takahashi, 2001). At P9/10, we observed no significant change in AMPAR-EPSC amplitude at 3.090 nA ± 0.858 nA for WT and 3.925 nA ± 0.575 nA for NR1-KDs (p=0.4373). After the onset of hearing at around P12, AMPAR-EPSCs increase in amplitude in the WT mice, and even more so in the NR1-KD mice. At P13/14, NR1-KD AMPARS-EPSC amplitudes, though not significantly, are 30.6% larger (p=0.14) at 7.089 nA ± 0.789 nA compared to the mean WT value of 5.427 nA ± 0.251 nA. The difference increases significance at P15/16 with the NR1-KD AMPAR-EPSCs 38.5% larger (p=0.0065) at 8.413 nA ± 0.347 nA compared to WT AMPAR-EPSCs at 6.071 nA ± 0.516 nA, and at P17/18 with it 26.6% larger (p=0.0138) at 13.208 nA ± 0.487 nA compared to a WT value of 10.434 nA ± 0.855 nA. This demonstrates that the decreased level of NMDARs at the synapse increases synaptic AMPARs only after the onset of hearing. The increase of AMPAR-EPSCs is possibly due to AMPARs physically having more space for insertion at the synapse when synaptic NMDARs are reduced. However, no such phenomenon is observed prior to the onset of hearing when the differences in NMDAR-EPSC amplitudes between NR1-KD and WT neurons are at its highest. More likely, NMDA receptors have an activity-dependent control of AMPAR insertion, triggered by external sound stimuli.
Figure 3.2 Slower AMPAR-EPSC kinetics are present prior to onset of hearing and larger current amplitudes were observed in mature NR1-KD calyx of Held-MNTB principal neuron synapse. Synaptic AMPAR-EPSCs were obtained by delivering a single stimulating pulse to the presynaptic axon while holding the MNTB principal neuron at −60 mV (example trace A & B for P9/10 and P17/18, respectively). The NR1-KD synapses have larger AMPAR-EPSCs after the onset of hearing (C). The AMPAR-EPSCs of the NR1-KD also had very slow rise (D) and decay (E & F) kinetics transiently at P9/10 compared to synapses of WT littermates. mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 4, 5, 4, 6, 6 neurons for WT, respectively, and n = 4, 6, 6, 5, 9 neurons for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001.
Previous experiments have shown that there is a subunit switch from GluR1 to GluR4 dominant composition in native synaptic AMPARs while GluR2 subunit is largely absent during development (Joshi & Wang, 2002), as manifested by strong inward rectification in current-voltage relationships the current-voltage relationship for AMPAR-EPSCs. By including spermine in the intracellular solution to prevent washout of endogenous spermine during recording, we examined current-voltage relationships of AMPARs in WT and NR1-KD synapses by delivering a single stimulating pulse at every 20 mV interval between ±60 mV V_h. As intracellular spermine inwardly rectifies currents of Ca²⁺ permeable AMPARs at positive potentials, measuring changes in I_[−40]/I_[+40] allowed us to infer the contribution of GluR2 if any to native AMPARs, which renders the receptor impermeable to Ca²⁺ and resistant to the effect of spermine. In this case, the closer the I_[−40]/I_[+40] ratio is to 1, the higher the proportion of GluR2-containing AMPARs is expressed. Apart from changes in amplitude after the onset of hearing, there are no significant changes in voltage dependence of AMPAR-EPSC between NR1-KD and WT calyx of Held synapses at any age group (S2). In addition, there were no observable differences in I_[−40]/I_[+40] ratios between NR1-KD and WT synapses at any age group, nor were there any developmental changes in this value, suggesting the AMPAR subunit composition remain unaltered in regards to the GluR2 subunit.

Since AMPAR-EPSCs develop to a larger and more rapid waveform, we also measured the kinetics of WT and NR1-KD AMPAR-EPSCs to establish any developmental effects of NMDAR reduction on the time course of AMPAR-EPSCs. We studied the time it takes for the EPSC to rise from 10% to 90% of its peak amplitude, as well as calculated the fast and slow decay time constants by fitting AMPAR-EPSC decay with a double exponential curve (Fig.3.2 D-F). Like previous experiments, we observed a decrease in both times during maturation of the synapse, in agreement with a development to more rapid AMPAR kinetics to maintain high synaptic fidelity (Joshi &
When comparing the WT synapse to the NR1-KDs, significant differences were only observed at P9/10 with the NR1-KD AMPAR-EPSC exhibiting much slower rise and decay kinetics (a 10-90 rise time of 0.420 ms ± 0.018 ms for NR1-KD compared to 0.276 ms ± 0.002 ms for WT, p=0.0012; a $\tau_{fast}$ of 1.692 ms ± 0.093 ms for NR1-KD compared to 0.651 ms ± 0.030 ms for WT, p=0.0001; and finally a $\tau_{slow}$ of 4.163 ms ± 0.360 ms for NR1-KD compared to 2.138 ms ± 0.163 ms for WT, p=0.0022). The slower AMPAR-EPSC kinetics was only present transiently at P9/10 as there were no differences observed beyond this age group. The widening of AMPAR-EPSCs can be attributed to a variety of presynaptic and postsynaptic factors, such as desynchrony of SV fusion that prolongs glutamate release, diffuse expression of AMPARs, slowed glutamate clearance from the synaptic cleft, or alterations in AMPAR subunit composition to that of slower kinetics.

To determine if the widening of AMPAR-EPSCs in the P9/10 NR1-KD was due to changes in postsynaptic receptor properties or alteration in presynaptic neurotransmitter (NT) release, we studied miniature (m) EPSCs in response to spontaneous presynaptic release events (Fig. 3.3 A). mEPSCs provide several clues as to the alterations that exist at quantal SV fusion events. By studying amplitudes of mEPSCs we can infer changes in the number of AMPARs, the amount of glutamate loaded in the SV; mEPSC rise and decay time is correlated to the activation and desensitization kinetics of AMPARs; while the mEPSC frequency is an indicator on the spontaneous release probability of SVs or the ability of AMPARs to detect quantal events by covering more area. We wanted to establish if there are any changes in the content of SVs in the NR1-KD calyx of Held synapse, so we measured mEPSC amplitude (Fig. 3.3 B). Though both WT and NR1-KD calyces demonstrate increasing amplitudes during maturation, a significant difference between the two groups were found only at P9/10 where mEPSCs of the NR1-KD were
Figure 3.3 **Studying spontaneous mEPSCs revealed major changes in the NR1-KD properties prior to the onset of hearing.** Recordings were obtained without any presynaptic stimulation for 3 minutes at –60 mV (example traces A). We observed regular developmental profiles between NR1-KD and WT synapses, with the exception of P9/10 (B & C). At this age group, amplitudes in the NR1-KD were larger than in the WT (B) and had a longer rise time (C). Decay time was also increased in the NR1-KD (Ea) with the distribution of decay time constants shifted to the right (Eb). Analysis of the inter-event intervals also shows a greater frequency of mEPSCs in the NR1-KD (D). mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 3, 5, 4, 6, 6 neurons for WT, respectively, and n = 4, 6, 6, 5, 7 neurons for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001; KS-test for inter-event interval at P9/10: p<0.001.
34.8% larger at 46.377 pA ± 1.97 pA compared to 34.40 pA ± 2.28 pA for WT mEPSCs (p=0.0105). The larger mEPSCs in the P9/10 NR1-KD is likely a result of an increase in glutamate release per SV fusion event.

In addition to being larger, NR1-KD mEPSCs at P9/10 are also slower with a mean 10-90 rise time of 0.3802 ms ± 0.0079 ms compared to 0.2677 ms ± 0.0178 ms for WT mEPSCs (p=0.0014) (Fig.3.3 C). Rise time for both groups declined with age, indicating maturation to a larger and more rapid mEPSC, contributing to the fast evoked AMPAR-EPSCs at mature synapses. We fit the decay of each mEPSC with a single exponential function (Fig.3.3 E). At P9/10, NR1-KD mEPSCs have larger decay time constants than WT mEPSCs, corresponding with a slower decay, peaking at 0.8 ms (mean of 0.98 ms ± 0.06 ms) for NR1-KD mEPSCs vs. 0.5 ms (mean of 0.61 ms ± 0.02 ms) for WT mEPSCs (p=0.0042). The slower rise and decay times of P9/10 NR1-KD mEPSCs are possibly a result of alterations in unitary AMPAR gating kinetics. This indicates a postsynaptic factor contributes to the phenotypes seen in AMPAR-EPSCs, suggesting slower EPSC kinetics to not be a result of changes synchrony of presynaptic SV release. Though the total number of AMPARs appears to be unchanged in the P9/10 NR1-KD MNTB neuron, as evidenced by no significant change in AMPAR-EPSCs at that age group, having a drastically lower expression of NMDARs may allow AMPARs to be more dispersed in the PSD. A more dispersed expression of AMPAR leads to an increase in detection of leftover glutamate in the synaptic cleft, and allows for even slower EPSC decay times than observed in WT mEPSCs.

By measuring the inter-event intervals between each mEPSC and plotting them on a cumulative histogram (Fig.3.3 D), we find the frequency of mEPSCs increases during development in both the NR1-KD and WT synapses (S3). There is a much higher frequency of spontaneous release at NR1-KD synapses at P9/10 and P17/18.
(Kolmogorov–Smirnov test, p<0.0001), indicating an increase in presynaptic release of SVs and/or an increase of detection of quantal events by a greater number or more widely dispersed postsynaptic AMPARs. The mean AMPAR-EPSC amplitude of NR1-KD calyces is not significantly larger than WT calyces at P9/10 even though the quantal amount of glutamate released is increased, as evidenced by the larger mEPSC amplitudes in the NR1-KD synapse. We suspect this is because there is a decrease in the number of synaptic vesicles, using the approximation: EPSC = number of synaptic vesicles in the RRP * probability of SV fusion * quantal glutamate. In addition, an increase in spontaneous release probability could also be the cause of a greater mEPSC frequency in the NR1-KD synapse.

3.3 NMDARs play a vital role in short-term depression and replenishment of SVs in the developing calyx of Held synapse

One of the vital features of the mature calyx of Held synapse is high synaptic fidelity during high frequency stimulation. By analysing the amplitudes of evoked currents during high frequency trains we further our understanding of how the synaptic drive of AP firing is maintained in the synapse. Short-term depression (STD), defined as the decrease in EPSC amplitude in response to repeated stimuli, can be accounted for by a variety of factors. Depletion or partial depletion of presynaptic vesicles results in smaller SV pool available for release in response to subsequent stimuli and lead to reduced EPSC amplitudes and greater depression; auto-inhibition of presynaptic Ca2+ channels by G-protein coupled receptors; inadequate clearing of glutamate from the synaptic cleft can leave more AMPARs desensitised for a longer period of time, resulting in lower AMPAR-EPSC amplitudes and severer STD; previously released Ca2+ may linger at presynaptic sites longer and cause refractory od release sites, exacerbating
depression. At the calyx of Held synapse, STD has been shown to be largely accounted for by a depletion of SVs, while desensitization only contribute to it in immature synapses but not mature synapses.

STD, as measured by the decreasing amplitudes of AMPAR-EPSCs during a 100 Hz 100 ms stimulation train (Fig.3.4), is more robust in the NR1-KD calyx of Held synapse at P9/10, decreasing to 10% of the original amplitude as opposed to 25% in the WT calyx (p=0.0218). During maturation, the level of STD using this protocol decreases in both the WT and the NR1-KD calyx (S4), however STD in the NR1-KD is significantly lower at P17/18, which depresses to 50% of the initial amplitude, compared to 35% in the WT (p=0.0264, Fig.3.4 C-H). When this protocol was repeated with 200 Hz (S5) and 300 Hz (S6) trains of 100 ms in duration, differences in STD between the NR1-KD and WT calyx was observed only in the initial ten EPSCs of the trains, in a similar pattern as seen during the 100 Hz 100 ms protocol (Fig.3.5). This surprising finding suggests the NR1-KD postsynaptically has a profound impact on STD, which is primarily of presynaptic origin in the calyx of Held-MNTB principal neuron synapse. In particular most differences are found in the initial segment of STD when there is relatively low accumulation of presynaptic Ca\(^{2+}\), suggesting an upregulation in the size of the readily releasable pool of SVs (RRP) and release probability.

Since STD is highly dependent on presynaptic release and recovery, we next investigated changes in the regulation of synaptic vesicles in NR1-KD synapses. We analysed quantified the steady state of EPSCs and paried-pulse ratios (PPRs), respectively. The steady state EPSCs was measured as the average amplitude of five AMPAR-EPSCs after STD is fully developed (or depletion of synaptic vesicles in the RRP) using a 300 Hz 100 ms trains (Fig.3.6 C-D). We observed a 48% larger amplitude in synapses at P17/18 in the NR1-KD calyx, with 1.458 nA ±0.161 nA steady state current, compared to the WT calyx, at 0.984 nA ±0.97 nA (p=0.0460).
Figure 3.4 Analysis of trains of stimuli demonstrate NR1-KD calyces have an exaggerated maturational trend of STD. A 100 Hz 100 ms train causes a depression of AMPAR-EPSCs throughout the duration of the train (example trace A & B for P9/10 and P17/18, respectively). By comparing the raw EPSC amplitudes over several age groups (C, D, E), we observed an enhancement of the developmental trend in the NR1-KD synapse. This was measured as a comparison of the final EPSC amplitude, indicated by dashed lines. This was also observable when EPSCs were normalized to the amplitude of the EPSC of the train (F, G, H). mean ± SEM; in age groups P9/10, P13/14, P17/18, n = 3, 4, 6 neurons for WT, respectively, and n = 4, 6, 9 neurons for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001.
Figure 3.5 Higher frequency trains show changes in STD in the NR1-KD synapse are only apparent in the initial segment of the train. Further analysis of STD using 200 Hz 100 ms trains still shows developmental alterations in STD (example trace A & B for P9/10 and P17/18, respectively), however the differences between NR1-KD and WT synapses are only observable within the first ten stimuli of the train for both the raw amplitudes (C & D) and amplitude normalized to the first EPSC in the train (E & F). Mean ± SEM; in age groups P9/10, P17/18, n = 3, 6 neurons for WT, respectively, and n = 4, 9 neurons for NR1-KD, respectively.
In immature calyces (P11/12), paired pulse amplitude ratios, measured as $A_2/A_1$, of NR1-KD synapses were visually lower than WT calyces, though not significantly so (Fig.3.6 A). However, PPRs of the mature (P17/18) NR1-KD calyx were 10% higher than the WT calyx at 3.3 ms ($p=0.0350$) intervals, and not significantly at 5.0 ms or 10.0 ms intervals ($p=0.1694$, $p=0.1020$ respectively) (Fig.3.6 B). Overall, PPRs increase during maturation in both the NR1-KD and WT synapses. Since the PPRs were $<1$ for all intervals and all measured age groups (S7), paired-pulse depression is predominant in the calyx of Held synapse. The strength of this depression has been correlated to the probability of synaptic vesicle release at presynaptic sites (Saviane et al., 2002). Therefore, there is a higher release probability in NR1-KD calyces prior to onset of hearing compared to WT synapses, a phenomenon that reverses significantly as the calyx matures. The relative paired-pulse depression observed between WT and NR1-KD synapses complements the results from STD studies, strongly indicating a cross-talk between postsynaptic NMDARs and presynaptic NT release machinery that alters Ca$^{2+}$-dependent SV fusion.

Measuring the speed of recovery of synaptic vesicles to the RRP provides further insights into the rate of synaptic vesicle recycling. Like measuring the steady state of SV replenishment, the RRP was first depleted using a 300 Hz 100 ms train. After waiting various intervals for the SVs to recover, we then depleted the RRP again to indirectly measure the amount of recovered SVs. We measured the recovery of synaptic vesicles by subtracting the mean steady state EPSC from individual EPSCs in both trains, then expressing ratio of cumulative amplitude of EPSCs of the 2nd vs 1st train. After the depletion of the RRP, recovery of EPSCs was enhanced in the NR1-KD calyx of Held (Fig.3.7 A). Throughout all age groups, NR1-KD calyces demonstrated more rapid recovery, specifically within the first 4 seconds after RRP depletion, with this difference growing as the calyx matures. At P11/12, NR1-KD neurons exhibited a 92.15% recovery
after a 2 second inter-train interval, compared to only 86.99% in WT neurons (Fig.3.7 B). There were no developmental changes observed in either slow or fast recovery time constants (S8), contrary to the results found in Joshi and Wang (2002), which illustrated a developmental hastening in recovery.

Discrepancy can be easily explained by significant differences between the acquisition and analysis protocols employed currently and in the aforementioned article. Namely, they used pairs of 100 Hz 100 ms stimulation strain as opposed to higher frequency of 300 Hz. The use of a higher frequency increases the Ca\textsuperscript{2+} build-up in the presynaptic terminal and allows for an analysis of a Ca\textsuperscript{2+}-dependent mechanism of recovery. In addition, they measured recovery as a proportion of the first EPSC in the second train to the amplitude of the first EPSC of the initial train. Through our method of measuring the summation of EPSCs in each train (after subtracting the steady state of replenishment) we measured the contribution of every SV present at the presynaptic terminal, allowing us to indirectly measure the total amount of SVs recovered.

As a result, the fast and slow time constants remained consistent throughout both group for all ages at approximately 0.100 s and 1.00 s respectively. However, the proportions of the recovery kinetics that follow $\tau_{\text{fast}}$ vs. $\tau_{\text{slow}}$ vary greatly between the NR1-KD and WT calyx, particularly after the onset of hearing. At P17/18, the WT calyx had recovery kinetics that adhered to $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ nearly equally (Fig.3.7 C). For the NR1-KD calyx of Held synapse, recovery is significantly faster with 62% of the recovery following fast kinetics and 38% following slow kinetics. These sets of experiments suggest that NR1-KD impacts both release and replenishment of SVs in the RRP. This also predicts that with the NR1-KD calyx of Held having increased recovery of SVs, particularly in the mature mouse, the ability of the MNTB neuron to fire APs at high frequency must be enhanced.
Figure 3.6 The enhanced maturational trend of PPD for the NR1-KD reflects the pattern observed in STD, with the mature NR1-KD synapse having a larger steady state of synaptic vesicle replenishment. Paired-pulse ratios were taken at intervals of 3.3 ms, 5.0 ms, and 10.0 ms for various age groups (example trace in insets of A & B for P11/12 and P17/18, respectively), except P9/10, which do not respond reliably at intervals of 3.3 ms. From this we found PPRs undergo a maturational shift similar to that seen in the 100 Hz STD study, with the NR1-KD following the same pattern of exaggerated maturation. The steady state of synaptic vesicle replenishment was calculated as the average amplitude of the last five EPSCs in a 300 Hz 100 ms train (example trace C). This number increases throughout development of both the NR1-KD and WT synapses (D), with the NR1-KD having a larger steady state of replenishment at P17-18. mean ± SEM; in age groups P11/12, P13/14, P15/16, P17/18, n = 4, 4, 5, 6 neurons for WT, respectively, and n = 5, 5, 5, 9 neurons for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001.
Figure 3.7 There is a faster recovery of SV in the RRP in NR1-KD synapses. In order to measure the recovery of the RRP, we depleted the SV using a 300 Hz 100 ms train. We repeated this depleting train after waiting various time intervals for the RRP to replenish (example trace with a 2 s inter-train interval A). The steady state of replenishment, measured by the average of the last five EPSC amplitudes of the second train, was subtracted from each EPSC, as denoted by the dashed line going across the traces. The summed amplitudes of the EPSCs in the second recovered train (amplitudes shaded in yellow) were expressed as a percentage of the summed amplitudes of the EPSCs in the initial fully replenished train (amplitudes shaded in blue). By plotting this percentage of the time of the inter-train interval, we can study the kinetics of SV recovery at various age groups (B & C for P11/12 and P17/18, respectively). Fitting the results with a double exponential cumulative probability curve with a $\tau_{fast} = 0.1$ s and $\tau_{slow} = 1.0$ s reveals faster recovery kinetics in the NR1-KD, especially in the mature age group, P17/18, mean $\pm$ SEM; in age groups P11/12, P17/18, n = 3, 4 neurons for WT, respectively, and n = 3, 3 neurons for NR1-KD, respectively; KS-test between NR1-KD and WT for P17/18 recovery curve p<0.001.
3.4 Attenuating NMDAR expression increases high fidelity postsynaptic firing in mature MNTB principal neurons

AP firing in the MNTB principal neuron in response to presynaptic trains of stimuli remains an important measurement of the synaptic fidelity of the calyx of Held synapse. The mature (P17/18) NR1-KD calyx of Held-MNTB principal neuron synapse has demonstrated decreased short-term and paired-pulse depression as well as higher SV recovery. All these factors implicate alterations in the firing properties of the NR1-KD MNTB neuron in response to presynaptic stimuli. To investigate any possible alterations, we studied the rate of spike failures during prolonged high frequency stimulation (100 Hz – 400 Hz, 400 ms trains) of the presynaptic axons. Though the ability to induce EPSCs remains unchanged between mature NR1-KD and WT calyces at various frequencies (Fig.3.8 A-C), this did not translate to the fidelity of AP generation at the calyx of Held when we switched to a current-clamp configuration (Fig.3.8 D-F). Mature NR1-KD calyces have a 20-fold lower rate of spiking failures at 100 Hz (0.40% ±0.33% failures for NR1-KDs vs. 7.8% ±4.2% for WTs; p=0.0839) and 200 Hz (1.4% ±0.99% for NR1-KDs vs. 27% ±10% for WTs; p=0.0235). At higher frequencies of stimulation, this difference was reduced to a 2.6-fold and 1.4-fold lower rate of spiking failures at 300 Hz (22% ±7% for NR1-KDs vs. 58% ±7% for WTs; p=0.0064) and 400 Hz (48% ±2% for NR1-KDs vs. 67% ±7% for WTs; p=0.0243), respectively. The increased spiking fidelity in the absence of increased EPSC fidelity observed in the NR1-KD synapse may either be a characteristic of the increased amplitude of AMPAR-EPSCs that drive AP generation in the MNTB principal neuron and/or the reduced NMDAR component that contributes to reducing the refractory period of AP firing and promotes subsequent firing. Since decreased STD in the NR1-KD synapse is only observed in the initial ten stimuli of the trains, it is not believed to play a large role in the enhanced AP firing we observed.
Figure 3.8 There is no alteration in fidelity of EPSCs in the NR1-KD synapse, but there is an increased fidelity of AP firing. We stimulated the presynaptic axon of the calyx of Held with various frequencies (100 Hz – 400 Hz) for a prolonged period of 400 ms to measure the sustained fidelity of the synapse. This was done in both the voltage clamp (example trace A) and current clamp (example trace D) configurations to obtain recordings of EPSCs and spiking, respectively. No differences were observed between the WT and the NR1-KD synapses in the number of failed EPSCs (B, insert a larger scale to view 100 Hz and 200 Hz), noting an increase
in the percentage of failures as the frequency is increased (C, insert a larger scale to view 100 Hz and 200 Hz). However, the NR1-KD synapses have a reduced number of AP failures compared to WT synapses (E, insert a larger scale to view 100 Hz). The percentage of failures for both groups of synapses increases with frequency and is much larger than EPSCs (F, insert a larger scale to view 100 Hz), concluding NR1-KDs more reliably fire APs with each EPSC. mean ± SEM; n = 6 neurons for WT, and n = 8 neurons for NR1-KD; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001.

Since the mature NR1-KD MNTB neurons have greater functional AMPARs with decreased STD, we next asked if these properties are associated with changes in intrinsic excitability of the MNTB neuron, which could also contribute to the increased AP firing we previously observed. Therefore, we studied the MNTB neuron membrane potential ($V_m$) and firing characteristics when injected with various current for 200 ms. Surprisingly, in response to current injections, mature (P17/18) NR1-KD neurons are more resistant to changes in voltage than WT calyces (p= 0.0397) (Fig.3.9 D). With a resting potential of approximately –70 mV, the $V_m$ of the NR1-KD MNTB principal neuron at the last 5 ms of each step plateaued to only –47 mV ± 2.7 mV with upwards of 1.0 nA of injected current. In contrast, the WT MNTB neuron further depolarized to a plateau potential of –37 mV ± 3.4 mV with the same current injections (p=0.0420 at 1.0 nA). In addition, the number of action potentials evoked during current injections was significantly lower in the NR1-KD neuron compared to WT neurons (Fig.3.9 C). When fitted with a Boltzmann function, half maximal AP generation was achieved with a mean $V_{mid}$ current of 0.20 nA in the WT MNTB neuron, whereas the NR1-KD MNTB neuron required a mean $V_{mid}$ current of 0.27 nA to achieve half maximal response. Despite the greater amount of current injected, the maximal number of APs generated by NR1-KD neurons (27.38 ± 6.23) was 32.7% lower than WT neurons (40.67 ± 0.26; p=0.1255). Also derived from the Boltzmann function, the slope factor ($V_c$) of the current-AP relationship for the NR1-KD neurons is 0.75 compared to 0.50 observed in WT MNTB neurons. The combined higher input-resistance to membrane potential change and fewer APs
generated suggests NR1-KD neurons are less excitable than their WT counterparts. Despite the decreased excitability of the neuron, mature NR1-KD synapses still have increased firing fidelity than WT synapses. This led us to conclude that stronger synaptic drive to NR1-KD MNTB principal neurons fully accounts for the increased fidelity in AP firing independent of its intrinsic excitability.

Figure 3.9 Mature NR1-KD MNTB principal neurons are less intrinsically excitable than neurons from WT littermates. In the current clamp configuration, we injected steps of current of various amplitudes for 200 ms to observe the spiking behaviour of mature (P17+) WT and NR1-KD MNTB principal neurons (example traces A & B, respectively; first seven steps shown). The peak number of spikes in the NR1-KD neuron is lower and occurs at a higher average injected current than the WT neurons (C). The relationship between current injected and action potentials fired was fit with a Boltzmann charge-voltage curve to obtain a Vmid and Vc. In addition, the plateau potential of the last 5 ms of the current step was measured and the NR1-KD was more resistant to changes in the membrane potential (D). The relationship between current and voltage was fit with a single exponential cumulative probability curve to obtain time constant tau. mean ± SEM; n = 3 neurons for WT, and n = 8 neurons for NR1-KD.
3.5 Knockdown of NR1 results in smaller calyces in immature synapses but does not affect the mature state

NMDAR activity has previously been shown to alter presynaptic and postsynaptic morphologies (Ultanir et al., 2007; Merriam et al., 2011). For this reason, we wanted to investigate the effect of chronic decrease of NMDARs on the presynaptic morphology of the calyx of Held synapse. This was achieved through fluorescent staining of the presynaptic neuron using a lipophilic Dil dye. Since morphology develops from a spoon-like structure to a more digitated state (Kandler & Friauf, 1993), we examined the presynaptic shape of both pre- and post-hearing onset WT and NR1-KD mice. When we investigated the morphology of the immature calyx (P11), we found the NR1-KD had a 7.7% smaller calyx of 18.22 μm ± 0.28 μm in diameter, compared to the 19.74 μm ± 0.34 μm diameter of the WT calyx (p=0.0015) (Fig.3.10 F).

Though the mean volumes and surface areas between WT and NR1-KD calyces aren’t significantly different (Fig.3.10 G-H), analysis of their distribution uncovers further details (Fig.3.10 I-J). The distribution of surface area for NR1-KD calyces reveals an abundance of calyces in the middle of the range. However, this did not translate to volume measurements, where the NR1-KD mice had a slight abundance of calyces with a larger volume. Combined with a smaller calyx diameter, measurements suggest a thicker presynaptic terminal. In addition, both the NR1-KD slice and WT slice had a great variety of presynaptic calyx morphologies (Fig.3.10 E). Both contained calyces that ranged from scoop-like morphologies and calyces that fully enveloped the MNTB neuron to calyces with a highly complex morphology with multiple digits and a large number swellings. Since the presynaptic terminal wraps around the MNTB neuron, a smaller calyx diameter in the NR1-KD group is likely the result of smaller MNTB principal neurons. Perhaps with large and significant decrease in the number of NMDARs on the
neuron surface the postsynaptic neurons have decreased in size to accommodate the reduced protein expression.

Surprisingly, analysis of the presynaptic terminal of the calyx of Held revealed few differences between the mature (P18) NR1-KD terminals and WT littermate’s terminals in mean calyx diameter (19.8 μm ±0.3 μm for NR1-KD vs. 20.1 μm ±0.5 μm for WT calyces), surface area (1857 μm² ±155 μm² for NR1-KD vs. 1758 μm² ±127 μm² for WT calyces), and volume (1010 μm³ ±121 μm³ for NR1-KD vs. 940 μm³ ±102 μm³ for WT calyces). Representing the clusters of calyces in a cumulative histogram show no alterations in the distribution of these properties, demonstrating, despite drastic reduction in NMDARs during development, overall morphology of the calyx remains unchanged in the mature NR1-KD mouse (Fig.3.10 B-D). This reveals that NMDARs play little role in the development of the presynaptic morphology development at the calyx of Held once the synapse is established in the post-hearing onset mice.

Intriguingly, preliminary evidence shows the NR1-KD slices at P11 had over twice the number of dyed calyces at 12.14 ±1.85 calyces/mm² in an approximately 50 μm deep window, as opposed to 5.55 ±0.13 calyces/mm² (p=0.0119) (S9). The increased density of calyces in the NR1-KD perhaps indicates an important role for NMDARs to prune the number of MNTB neurons. Alternatively, an apparent increase in the number of MNTB principal neurons could be a compensatory mechanism to overcome the dramatic decrease in NMDAR expression in each neuron, or could merely indicate a difference in the extent of staining of the presynaptic terminal.
Figure 3.10 Though there is an initial decrease in the diameter of NR1-KD calyces, they mature to a presynaptic terminal that is morphologically indifferent than WT calyces. Imaging of the presynaptic morphology was obtained through the anterograde lipid staining of the globular bushy cells using DiI crystals inserted into the aVCN of brains preserved in PFA (A). A z-stack composition of the images allowed for a 3-D reconstruction of the presynaptic terminal. Using the reconstruction we found there were no differences between the morphology of the mature (P18) WT (example images B) and NR1-KD (example images C) presynaptic terminals, with both illustrating similar distribution between morphological classifications as previously determined in Grande & Wang (2011) (D). No such classification exists for the immature (P11) morphology, but calyces were observed under several stages of development and fenestration in both groups (example images E). However, the NR1-KD does have a smaller calyx diameter than the WT at the immature age group (F), and though mean surface area and volume remain...
unaltered (G & H), the distribution of the surface area reveal a higher frequency of NR1-KD synapses at the mid-point than WT synapses (I). No such alteration in distribution was observed in the volume data (J). mean ± SEM; in age groups P11, P18, n = 14, 15 calyces for WT, respectively, and n = 18, 18 calyces for NR1-KD, respectively; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001; scale bar = 530 µm (A) and 10 µm (B, C, E).

3.6 Reduction of NMDARs do not alter sound-evoked auditory brainstem responses

Due to the role NMDARs play in synaptic development, we were curious as to whether a global reduction in NMDAR expression would alter acoustic transmission, specifically in the auditory brainstem. To investigate the role of NMDARs in sound detection we performed in vivo ABR recording in post-hearing onset WT and NR1-KD mice. The sound level threshold to elicit clear ABR waves varied greatly between litters of matured (P15+) mice. However, no significant differences in hearing threshold were observed between NR1-KD mice and their WT littermates. At audible sound levels, five unique peaks were identified in the ABRs of the mice, corresponding to synaptic transmission at various points along the auditory pathway (Fig.3.11 A). Waves I and II are generated by the peripheral and central portions of cranial nerve VIII, respectively. Wave III is generated by the cochlear nucleus, wave IV by the SOC, including the calyx of Held synapse, and wave V by the lateral lemniscus. The average amplitude of each peak did not differ between NR1-KD mice and WT mice when comparing recordings of identical sound levels, with the peak amplitudes generally occurring at waves 2 and 3 under all conditions (Fig.3.11 B).

Measuring the descending slope of each wave yielded high variability and no significant differences between ABRs from WT and NR1-KD mice. In addition, analysis of the latency of waves demonstrated no difference in timing of synaptic transmission.
NR1-KD had normal transmission of acoustical stimuli in the brainstem. Delivering a click of sound during in vivo recordings of ABRs from anaesthetized P15+ mice results in five distinct waves as the auditory stimulation is transmitted along various stages in the auditory brainstem circuitry (example traces A). When comparing responses from NR1-KD mice and T mice, we observed no alterations in the peaks of the waves, as measured from the previous trough, no the latency at which the peak occurs (B). No alterations in latency were observed at any sound level of the auditory stimulus (C), mean ± SEM; n = 6 mice for WT and n = 6 mice for NR1-KD.
between the NR1-KD and WT auditory pathway when comparing recordings from identical sound intensity levels (Fig.3.11 C). We repeated the comparison of all the aforementioned ABR features from minimum dB level needed to achieve a response for each mouse and, once again, no trends or significant differences could be found between the WT and NR1-KD mice. This illustrates that, though NMDARs are drastically reduced during development, there are no significant alterations in acoustical transmission that would either enhance or deteriorate the brainstem’s response to auditory stimuli, attesting the remarkable stability of classical sensory systems like the auditory brainstem.
CHAPTER FOUR: Discussion

4.1 Summary of major findings

We have shown a global reduction of functional NMDARs leads to several developmental changes in the synaptic properties of the calyx of Held (Fig. 4.1), most surprisingly, in short-term and paired-pulse depression in response to high frequency trains and recovery from synaptic depression, which are usually considered as presynaptic phenotypes. Contrary to the classical thinking that NMDARs are important for activity-dependent recruitment of AMPARs into synapses, we found the opposite: from after the onset of hearing P11/12 to fully mature NR1-KD calyx of Held synapse, there is no deficit, but instead a significant increase in AMPAR-EPSC amplitude and spiking fidelity. This alteration in synaptic function is not accompanied by any dramatic changes in the mature presynaptic morphology or acoustical transmission as measured by in vivo ABR wave properties. We suggest that minimal NMDARs are sufficient for initial buildup of synaptic structure and function at the calyx of Held synapse, and that after synapse formation NMDARs instead act as maturation brake through a negative feedback system, so that a reduction of NMDARs promotes development of both presynaptic and postsynaptic functions.

4.2 Reducing NMDARs in glutamatergic synapses

Genetic intervention successfully reduced NMDAR currents and, by extension, functional NMDAR expression in the MNTB principal neuron, particularly a large decrease of receptors located at postsynaptic densities, with less reduction of NMDAR function at perisynaptic sites of the MNTB-calyx of Held synapses. This reduction in
Figure 4.1 NMDAR function is believed to alter the calyx of Held – MNTB principal neuron synapse function through both postsynaptic and presynaptic modifications. Our results suggest NMDARs alter the synapse differently throughout development. Overall, NMDARs are implicated in promoting their own insertion into the PSD near synaptic sites. Possibly due to a Ca\(^{2+}\)-dependent process, NMDARs also appear to control AMPAR insertion as well as promote changes in early subunit composition, which alter gating kinetics. In addition, our results show that postsynaptic NMDARs likely have presynaptic functions, which include controlling the number of SVs and the amount of glutamate per vesicle. NMDARs also seem to decrease the rate of recovery of the SVs in the RRP during high frequency stimulation. All this changes suggest NMDAR function actually decreases the ability of the postsynaptic neuron to fire APs in response to presynaptic stimulation. (black arrows indicate flow of ions; red indicates processes affected by NMDAR function)
NMDARs induces several changes in the functional properties of the calyx of Held, such as slower decay kinetics of NMDAR-EPSCs in the NR1-KD calyx of Held, an increase in postsynaptic AMPAR-EPSCs and synaptic fidelity in mature mice, initial lag in AMPAR kinetics, alterations in short term depression and recovery during maturation, and the size of the calyx in the immature P11 synapse.

NMDARs are highly involved in development and have long been shown to play a vital role in long-term synaptic plasticity in an activity-dependent manner in the hippocampus (reviewed in Bliss & Collingridge, 1999 and Malenka & Bear, 2004). Adesnik et al. (2008) performed a mosaic deletion of NR1 expression in vitro in slices containing hippocampal cells and in vivo in embryos of a floxed NR1 mouse in a similar effort to determine the effect of NMDARs on synaptic maturation during a 12-17 day period. They similarly concluded NMDARs play a significant role in development, stating the receptors are involved in preventing premature synaptic maturation and inhibits synaptic unsilencing.

As NMDARs can act as a coincidence detector, the role of NMDARs to inhibit synaptic unsilencing is dependent on synaptic activity and consequently reliant on postsynaptic Ca^{2+} influx. A point mutation Grin1^{N598R} allele codes for NR1′ subunits that renders NMDARs insensitive to Mg^{2+} blockage and decreases Ca^{2+} permeability (Burnashev et al., 1992b; Mori et al., 1992), thereby eliminating NMDAR’s role as a coincidence detector and abolishing all activity-dependent forms of long-term synaptic plasticity without affecting total NMDAR expression. Mice that are heterozygotes for this allele were viable and survive to adulthood, whereupon 10% of NMDARs have at least one mutant NR1-r subunit (Behe et al., 1995). Despite the relatively low percentage of affected NMDARs in these heterozygous mice, long-term potentiation (LTP) is abolished in the dentate gyrus, while not affecting LTP in the CA1 region of the hippocampus (Chen et al., 2009). Spiking was impaired in the granule cells of the dentate gyrus and
mutated mice performed poorly in spatial learning tests such as the Morris water navigation task and place recognition in the annular water maze test. Therefore, even minimal alterations in NMDAR function can have profound effects on synaptic signalling and brain function in a region-specific fashion.

4.3 AMPARs and fidelity of spiking in mature calyces

Chronically reducing NMDARs increased postsynaptic AMPARs in the matured calyx of Held synapse. The mature neuron of the NR1-KD was less excitable than the WT littermate, characteristic of fewer NMDARs present for a persistent depolarisation of the membrane, however, an increase in AMPAR-EPSC amplitude resulted in greater synaptic fidelity when translated to post synaptic spike generation. We have previously shown a positive relationship between AMPAR-EPSC amplitude and synaptic fidelity of the calyx of Held in earlier studies in this lab (Joshi et al., 2004; Yang et al., 2011). Depleting GluR4 subunits from the MNTB neuron resulted in the opposite effect observed in this study. In the GluR4-KO, a decrease in AMPAR-EPSCs was correlated with a decrease in synaptic fidelity by a measured increase in the percentage of spiking failures during stimulating trains (Yang et al., 2011). In addition to the increase in AMPAR amplitude and synaptic fidelity in the mature NR1-KD calyx, we also observed and accompanying decrease in short-term depression, which is likely important for sustaining high-frequency firings.

Our observations in the mature NR1-KD mirror the results previously published on the hippocampus. Just as we have shown an increase in AMPAR-EPSC amplitude in the mature NR1-KD calyces, mosaic depletion of hippocampal NMDARs results in an increase in postsynaptic AMPAR currents (Adesnik et al., 2008). An increase in mEPSC frequency without changing amplitude was observed, much like the mature NR1-KD
calyx. An NMDAR rescue in the hippocampus decreased AMPAR-EPSC amplitudes, resulting in the conclusion that NMDARs restrict the number of functional synapses in hippocampal pyramidal neurons, which likely contributes to the lower fidelity of synaptic transmission in WT calyces. This is in contrast to afore mentioned NR1<sup>T</sup> subunit-containing mouse synapses, which demonstrated impaired LTP in the dentate gyrus of the hippocampus, suggesting an important role of NMDARs to recruit postsynaptic receptors such as AMPARs (Chen <i>et al.</i>, 2009). However, this mutation had no effect on the expression of other neurotransmitter receptors in whole brain membrane protein fractions (Rudhard <i>et al.</i>, 2003).

Different NMDAR subunits also have different effects on synaptic plasticity. NR2B subunits significantly contribute to LTP compared to NR2A via a higher affinity to binding to calmodulin kinase 2 (CaMKII; Barria & Malinow, 2005) and limit the incorporation of AMPARs in developing cortical synapses (Hall, Ripley & Ghosh, 2007). During maturation of the calyx of Held synapse, NR2B subunits are downregulated while NR2C subunits are inserted (Futai <i>et al.</i>, 2001; Steinert <i>et al.</i>, 2010), suggesting a developmental alteration in NMDAR-dependent synaptic plasticity and posing a potential mechanism to explain the upregulation of postsynaptic AMPARs as the synapse matures. In NR1-KDs, NR2A and NR2B subunit expression in the striatum, prefrontal cortex, and hippocampus was also reduced to around 10% of WT expression, however, the reduction of NR2B proteins in each of these regions was more robust than NR2A proteins (Ramsey <i>et al.</i>, 2008). If this trend is also true in the MNTB, the NR1-KD calyx has an even further reduced NR2B composition. This may explain the increased AMPARs in the mature NR1-KD MNTB principal neuron and possibly imply a compromised ability to induce LTP.
4.4 Synaptic organization and EPSC kinetics

The drastic decrease of NMDARs in the immature MNTB neuron is likely to have significant effects on the postsynaptic organization of macromolecules at the calyx of Held. Since most of these proteins are essential in neurotransmission, synaptic transmission in NR1-KD mice has greatly modified properties. The decay of the mEPSCs was 40% slower in the pre-hearing onset P9/10 NR1-KD calyx. However, evoked EPSCs in the NR1-KD were even slower with as 160% - 220% larger tau, indicating more than just slower deactivation and desensitisation kinetics of AMPARs. Like the NMDARs, this can attributed to a more dispersed expression of AMPARs in the NR1-KD calyx of held or, alternatively, a more desynchronised release of SVs (Diamond & Jahr, 1995).

In connection with a possibility of a more dispersed and free expression of AMPARs in the PSD, hippocampal and cultured neurons demonstrated that high mobility of AMPARs allow desensitised AMPARs to move away from sites adjacent to presynaptic active zones and leads to a replenishment of inactivated AMPARs. The new AMPARs essentially allows for increased recovery of synaptic currents during multiple stimulating events (Heine et al., 2009). Though this was measured as a paired-pulse depression, a property that we found was actually slightly decreased in the immature NR1-KD calyx, the inter-pulse interval they used was 50 ms, a time interval that is better matched with our recovery experiments. In this case, our results for recovery in the NR1-KD calyx for most age groups agree with the profile of a synapse with highly mobile and dispersed postsynaptic AMPARs. This indicates the faster recovery kinetics of AMPAR-EPSCs during high frequency trains may not be solely presynaptic in nature as previously believed to be true in mature calyx of Held synapses. The mechanism behind faster recovery in NR1-KD synapses can be clarified by repeating the experimental
protocol while introducing cyclothiazide (CTZ) to the extracellular solution to prevent AMPAR desensitization. If there is a postsynaptic mechanism by which recovery in the NR1-KD synapses are enhanced, via either reduced AMPAR desensitization or increased replenishment of desensitized AMPARs, then CTZ will abolish all or part of the differences in recovery kinetics between the NR1-KD and WT synapses.

AMPAR-EPSC kinetics is also determined by the subunit composition of AMPARs at postsynaptic sites. In particular, the inclusion of a GluR4 subunit accelerates the gating properties of the receptors in the rat hippocampus (Geiger et al., 1995), and in the calyx of Held synapse (Yang et al., 2011) and endbulb of Held in the auditory brainstem (Wang et al., 1998). There is a developmental increase in GluR4 subunit mRNA in the calyx of Held synapse (Koike-Tani, Saitoh & Takahashi, 2005). Since this mechanism pre-exists in the calyx, NMDARs perhaps play a role in the early recruitment of GluR4-containing AMPARs in the immature calyx, also resulting in rapid AMPAR-EPSC and mEPSC kinetics in the WT calyces.

We have also found the immature calyx of Held had a greater frequency of spontaneous EPSCs. The increased frequency of mEPSCs observed could represent an increase in spontaneous fusion events brought forth by a higher number of synapses or active zones, such as the previously documented increase in mEPSC frequency in NR1-depleted hippocampal cells (Adesnik et al., 2008) and in NR1-null cortical pyramidal neurons (Ultanir et al., 2007). This observation has great consequences in the calyx of Held as it strongly implies a postsynaptic regulation of the organization of presynaptic components and synaptic vesicle release machinery. Analysis of PPRs suggests a trend towards a larger release probability in immature NR1-KD calyces, likely accounting for the increase in mEPSC frequency.
4.5 NR1-KDs ‘hyperdevelop’ calyces, with NMDARs acting as a maturation brake

NMDARs play a great role in the maturation of the calyx of Held. Due to the enhanced development of the NR1-KD calyx, the presence of NMDARs in the calyx of Held acts as a brake to control the rate and extent of maturation. This was observed in studying the short-term depression and paired pulse ratio of the calyx, forming a calyx with more functional AMPARs, increased synaptic fidelity and lower SV release probability. The greatest alterations of STD in NR1-KD synapses were in the initial ten stimuli of trains of any frequency, indicating a Ca\(^{2+}\)-dependent process.

The rapid recovery phase of short-term depression may reflect recovery from postsynaptic receptor desensitisation either through AMPAR alterations or changes in glutamate clearance, amount of residual Ca\(^{2+}\) in the presynaptic terminal, or release probability, all of which affects subsequent EPSCs (Dittman & Regehr, 1998). To determine which mechanism leads to alterations in STD in the NR1-KD calyx, future experiments by loading the presynaptic terminal with slow-acting Ca\(^{2+}\) buffer, EGTA, to reduce intracellular Ca\(^{2+}\) without stopping SV release, are needed. On the other hand, AMPAR desensitisation can be blocked by perfusing the slice with cyclothiazide (CTZ) to determine if AMPAR desensitisation plays a role in the alterations of STD.

NMDARs have previously been described as a factor that prevents premature synaptic maturation (Adesnik et al., 2008). This vital role is dependent on activity to engage NMDAR function, which explains why the effect of NMDARs in the calyx of Held synapse is more profound after the onset of hearing at P12. The enhanced maturation observed in the NR1-deleted pyramidal neurons was decreased via a rescued expression of WT NR1 subunits, demonstrating the synaptic changes observed was in fact a direct effect of NMDARs (Adesnik et al., 2008).
4.6 Alteration of presynaptic morphology and its effect on calyx function

The reduction of NMDARs did not affect the development of the calyx to a mature, digitated structure. However, several differences were observed in the immature calyx presumably as a downstream result of significantly decreased NMDAR expression. A smaller presynaptic calyx coincides with the marked decrease in postsynaptic receptors. Though diameter is altered, the mean surface area and volume are not. There has been a correlation between NMDARs and presynaptic morphology and development made in previous studies (Ultanir et al., 2007; Sceniak, Berry & Sabo, 2012, respectively). Genetic deletion of NR1 subunits in the cortex of mice reduced total NMDAR expression and lead to a 75% larger presynaptic bouton along with postsynaptic dendritic spine changes. While blocking NMDAR activity reduces synaptic vesicle and active zone protein recruitment to the presynaptic terminal in neocortical neurons. Since the size of the presynaptic calyx and MNTB neuron are only altered at P11 when the percentage of NMDAR reduction is the highest, this raises the possibility that lower spontaneous NMDAR activity reduces the size of the synaptic terminals.

It was previously demonstrated, in other areas of the brain, that NMDARs play a role in postsynaptic restructuring. Indeed, NMDAR activity is known to affect dynamic microtubules in dendritic spines in mice, causing an enlargement of the dendritic spines and resulting in LTP (Merriam et al., 2011), while eliminating NR1 expression in cortical pyramidal neurons enhance postsynaptic density area (Ultanir et al., 2007). No such change in size is found in the mature MNTB, an observation supported by the unaltered spine morphology and density in NR1-depleted mouse hippocampal slices (Adesnik et al., 2008). Alternatively, contradictory results were found by Alvarez, Ridenour, and
Sabatini (2007), showing knocking down NR1 in the hippocampus destabilizes synaptic structure and leads to loss of dendritic spines. This loss was attributed to the necessity of postsynaptic NMDARs to stabilize synaptic structure and maintain functional excitatory synapses.

Analysis of the surface area data of the immature (P11) NR1-KD calyces show there was an abundance of presynaptic terminals in the mid-point of the accumulated data range. In previous studies, mature calyces in the middle range of surface area were generally classified as morphological type 2 with a moderate number of swellings (Grande & Wang, 2011). However, this particular classification system has not yet been applied to immature calyces and fails to describe morphological observations at that age. We have previously investigated the correlation between calyx morphology and function (Grande & Wang, 2011). A more simple and open calyx, such as the type 1 calyx, is associated with an exaggerated short-term depression in response to trains and a lower paired-pulse ratio, a synaptic phenotype we have observed in the immature NR1-KD calyces. This was reversed in mature NR1-KD calyces, which is typical of type 3 calyces. As there are no morphological changes in the mature NR1-KD calyx, the reduced STD and PPD observed could not be attributed to morphological alterations, and more likely reflect subsynaptic changes in Ca\(^{2+}\) influx and downstream fusion process.

### 4.7 Pruning of the calyx of Held synapse

Higher NMDAR activity has been linked to hearing impairments through neurotoxicity in the cochlear nucleus (McDonald et al., 1998; Li et al., 2010). Since preliminary evidence suggests a much greater density of calyces were in the NR1-KD MNTB compared to the WT MNTB, perhaps the elevated NMDAR expression just prior
to hearing plays a role in the early phase of pruning synapses to the smaller number of neurons found in the adult MNTB. Controlled excitotoxic neuronal loss was shown to prune synapses in the frontal cortex and improve neural networks, contributing to the perceptual ability (Hoffman & McGlashan, 1997). This neurotoxicity has been attributed to excessive glutamatergic signalling (Olney, Sharpe & Feigin, 1972), particularly through activation of NMDARs (reviewed in Michaelis, 1998). NMDAR-driven Ca\(^{2+}\) influx into the neuron activates calmodulin kinases (CaMK), which in turn activated transcription factors that lead to necrosis and apoptosis. Additionally a large Ca\(^{2+}\) influx enhances mitochondrial electron transport, producing reactive oxygen species and adding to the oxidative stress of the neuron.

The MNTB undergoes a developmental pruning of principal neurons, resulting in fewer synapses between the calyx of Held and principal neurons in the mature mouse brain. NMDAR-dependent programmed cell death through mechanisms similar to excitotoxic apoptosis may play a role in this synaptic pruning during maturation of the MNTB, resulting in delayed pruning observed in the immature NR1-KD MNTB. Synaptic pruning eventually occurred, since the density of calyces in the adult NR1-KD MNTB did not differ from the WT. Unpublished studies provide evidence early calyx of Held formation is reliant on the binding of bone morphogenetic proteins (BMP) to BMP receptors 1a and 1b (Xiao, 2011). Clarification of these preliminary observations will be aided greatly by increasing the number of mice and using a 4',6-diamidino-2-phenylindole (DAPI) staining to label the nucleus of the MNTB principal neuron and obtaining more accurate density counts that will help determine if there really are more neurons in the NR1-KD. In addition, experiments involving chronic block of NMDAR activity or Ca\(^{2+}\) permeability in the MNTB neurons of mice may help determine if pruning is a result of a programmed excitotoxic-like apoptotic event.
4.8 Acoustic transmission

There were no significant alterations in acoustical transmission in the NR1-KD, leading to the conclusion that though NMDARs are critical for the development of individual synapses in a network of neurons, they do not affect audition as a whole. This may have significant implications in the stability of ancient sensory processes that retain their function and development even in the face of altered synaptic plasticity. Due to a frequency-dependent enhancement of synaptic fidelity, it would be interesting to observe the ABRs obtained from exposing the mice to a sound stimulus of varying pitch. An attempt to attain these recordings were made, however no significant traces were obtained due to deterioration of the acoustical signalling as a result of the prolonged anaesthetic treatment of the mice. Previously, acoustical studies in the NR1-KD show the mice have an exaggerated startle response to auditory stimuli (Duncan et al., 2006) and reduced sensory processing and selective attention (Bickel, Lipp & Umbricht, 2006, 2008; Halene et al., 2009), indicating alterations in the processing of acoustic stimuli downstream on the auditory brainstem.

NMDARs do play a role in the shape and timing of mammalian ABRs. In mice it was determined global mutation of the NR2A subunit had no effect on the hearing threshold of adult mice in response to clicks of stimuli (Munemoto et al., 1998). However, the NR2D global mutation significantly impaired hearing in adult mice. There is little to no expression of NR2D subunits in the calyx of Held (Steinert et al., 2010). However, they are expressed in other regions of the auditory circuit, which contribute to the waves of electric potential in ABRs (Munemoto et al., 1998). Since click-induced ABRs were unaffected in NR1-KD mice, the relative expression of NR2D subunits are likely unaffected by the mutation. Considering NR2D-containing NMDARs have slow kinetics,
a further test of their relative expression could be accomplished by studying ABRs in response to stimuli of various frequencies to determine if there is a frequency-dependent impairment in acoustic transmission in the auditory brainstem.

High NMDAR induced calmodulin activity in the cochlear nucleus of neonatal rats has generally been associated with neurotoxicity, deterioration of ABR thresholds and increase of peak wave latencies (McDonald et al., 1998; Li et al. 2010). This is the believed mechanism by which hyperbilirubinemia affects hearing in children. Although there is possibly reduced NMDAR-dependent cell death in the immature NR1-KD MNTB, depletion of synapses was not observed in the mature NR1-KD MNTB leaving ABRs unaffected in the post-onset of hearing mice.

4.9 Concluding remarks and outlook for future studies

The NR1-KD has provided great insights to the role of NMDARs in the developing calyx of Held. In particular, our evidence suggests NMDARs may potentially act as a control to inhibit and regulate synaptic maturation. Mechanisms by which NMDARs affect STD and PPD have yet to be resolved, but it is likely that NMDARs play important role in cross-talk between pre- and postsynaptic compartments via physical or diffusible retrograde signals to regulate development of short-term plasticity. Exposing the presynaptic terminal to Ca$^{2+}$ buffer EGTA will determine the Ca$^{2+}$-dependence of synaptic phenotypes of the NR1-KD calyx of Held synapse, while including CTZ in the extracellular recording solution will elucidate possible contributions of altered AMPAR desensitization properties. Due to different properties of NMDAR subunits, immunohistochemical staining or immunoblotting of different NR2 subunits expressed in the membrane will aid in determining if there are any alterations in relative incorporation of specific subunits into the NMDAR structure. While investigating into the possible
NMDAR and Ca\textsuperscript{2+}-dependence of MNTB principal neuron pruning may reveal yet another role NMDAR activity plays in the developing auditory brainstem.

To confirm all our reported observations are due to the development-dependent decrease in NMDAR function and not a direct result of acute reduced NMDAR currents, a repeat of the full electrophysiological protocol in the WT using bath application of an NMDAR antagonist such as (2R)-amino-5-phosphonovaleric acid (APV) is warranted. Application of this blocker was previously shown to increase spiking fidelity in young calyx of Held-MNTB principal neuron synapses (Futai et al., 2001), but detailed studies on how it effects AMPAR-EPSCs have yet to be performed. This will determine if synaptic properties change with little to no input from NMDARs and may alter the interpretation of our NMDAR hypofunction model. Finally, though there is no evidence to suggest the presence of NMDAR current in the presynaptic terminal, there is still reduced NMDAR function in the soma of the presynaptic neurons, the globular bushy cells. The anterograde effects of this reduced NMDAR function are unlikely the cause of our presynaptic observations but cannot be ruled out. Therefore, studies on the influence of NMDARs on the calyx would greatly benefit from a total knockout of NR1 specifically expressed in the MNTB principal neuron alone.
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APPENDIX I
Supplementary Figures

A  NMDA-EPSCs at P9/10 (±60 mV)

B  I-V Curve at P9/10

C  I-V Curve at P11/12

D  I-V Curve at P13/14

E  I-V Curve at P15/16

F  I-V Curve at P17/18

S1 Compiled NMDAR-EPSC current-voltage relationships for all age groups We delivered a single stimulating pulse to the presynaptic axon while holding the MNTB principal neuron at various holding potential between ±60 mV (example traces A). By measuring the amplitude of the slow NMDAR component of the EPSC we compiled current-voltage curves for several age groups throughout the development of the synapse (B - F). The reversal potential remains largely consistent between NR1-KD neurons and WT neurons in any age group. Apart from decreased amplitude, no changes were seen in the shape of the curves. mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 3, 5, 4, 4, 5 neurons for WT, respectively, and n = 4, 6, 6, 5, 10 neurons for NR1-KD, respectively
Compiled AMPAR-EPSC current-voltage relationships for all age groups

We delivered a single stimulating pulse to the presynaptic axon while holding the MNTB principal neuron at various holding potential between ±60 mV (example traces A). By measuring the amplitude of the rapid AMPAR component of the EPSC (measured as the peak amplitude within the dashed lines in traces A) we compiled current-voltage curves for several age groups throughout the development of the synapse (B - F). There was no observable change in the reversal potential between NR1-KD neurons and WT neurons in any age group. Analysis of the absolute values of the current at –40 mV divided by the current at +40 mV allows us to indicate alterations in the inward rectification of positive current, characteristic of GluR2-absent synapses (G). There are no differences between the NR1-KD synapse or WT synapse observed, nor are there any developmental alterations in this value. mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 4, 5, 4, 6, 6 neurons for WT, respectively, and n = 4, 6, 6, 5, 9 neurons for NR1-KD, respectively.
S3 mEPSC frequency data extended through all age groups shows a developmental increase in frequency

Recordings were obtained without any presynaptic stimulation for 3 minutes for each neuron at –60 mV (example traces A). Analysis of the inter-event intervals through all age groups shows a greater frequency of mEPSCs in the NR1-KD (D - F). mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 3, 5, 4, 6, 6 neurons for WT, respectively, and n = 4, 6, 6, 5, 7 neurons for NR1-KD, respectively; KS-test for inter-event interval at P9/10, P17/18: p<0.001.


**S4 STD during 100 Hz 100 ms trains data extended through all age groups** A 100 Hz 100 ms train causes a depression of AMPAR-EPSCs throughout the duration of the train. By comparing the raw EPSC amplitudes over several age groups (A – E; group a) of the NR1-KD and WT calyces, we observed an enhancement of the developmental trend in the NR1-KD synapse. This was also observable when EPSCs were plotted in relation to the amplitude of the EPSC of the train (group b). mean ± SEM; in age groups P9/10, P11/12, P13/14, P15/16, P17/18, n = 3, 5, 4, 6, 6 neurons for WT, respectively, and n = 4, 6, 6, 5, 9 neurons for NR1-KD, respectively.
A 200 Hz 100 ms train causes a depression of AMPAR-EPSCs throughout the duration of the train. By comparing the raw EPSC amplitudes over several age groups (A–D; group a) of the NR1-KD and WT calyces, we observed the enhancement of the developmental trend in the NR1-KD synapse is mostly limited to the first ten stimuli in the trains. This pattern was absent when EPSCs were plotted in relation to the amplitude of the EPSC of the train (group b). P9/10 was omitted due to high number of failures to induce EPSCs at 200 Hz frequency. mean ± SEM; in age groups P11/12, P13/14, P15/16, P17/18, n = 3, 4, 6, 5 neurons for WT, respectively, and n = 4, 4, 5, 7 neurons for NR1-KD, respectively.
S6 STD during 300 Hz 100 ms trains data extended through all age groups A 300 Hz 100 ms train causes a depression of AMPAR-EPSCs throughout the duration of the train. By comparing the raw EPSC amplitudes over several age groups (A – D; group a) of the NR1-KD and WT calyces, we still observed the enhancement of the developmental trend in the NR1-KD synapse is limited to the first ten stimuli in the trains and is absent when EPSCs were normalized to the amplitude of the EPSC of the train (group b). P9/10 was omitted due to high number of failures to induce EPSCs at 300 Hz frequency. mean ± SEM; in age groups P11/12, P13/14, P15/16, P17/18, n = 3, 4, 5, 6 neurons for WT, respectively, and n = 3, 4, 5, 9 neurons for NR1-KD, respectively.
S7 PPR data extended through all age groups illustrating an increase in PPR through development

Paired-pulse ratios were taken at intervals of 3.3 ms, 5.0 ms, and 10.0 ms for various age groups (A - D), except P9/10, which do not respond reliably at intervals of 3.3 ms. From this we found PPRs undergo a maturational shift similar to that seen in the 100 Hz STD study, with the NR1-KD following the same pattern of exaggerated maturation. mean ± SEM; P11/12, P13/14, P15/16, P17/18, n = 3, 4, 5, 6 neurons for WT, respectively, and n = 3, 4, 5, 9 neurons for NR1-KD, respectively.
Recovery of synaptic vesicles using 300 Hz 100 ms trains for all age groups

In order to measure the recovery of the RRP, we depleted the SV using a 300 Hz 100 ms train. We repeated this depleting train after waiting various time intervals for the RRP to replenish. The steady state of replenishment, measured by the average of the last five EPSC amplitudes of the second train, was subtracted from each EPSC. The summated amplitudes of the EPSCs in the second recovered train were expressed as a percentage of the summated amplitudes of the EPSCs in the initial fully replenished train. By plotting this percentage of the time of the inter-train interval, we can study the kinetics of SV recovery at various age groups (A - D). P9/10 was omitted due to high number of failures to induce EPSCs at 300 Hz frequency. Fitting the results with a double exponential cumulative probability curve with a $\tau_{\text{fast}} = 0.1$ s and $\tau_{\text{slow}} = 1.0$ s reveals faster recovery kinetics in the NR1-KD, especially in the mature age group, P17/18. Mean ± SEM; in age groups P11/12, P17/18, $n = 3$, 4 neurons for WT, respectively, and $n = 3$, 3 neurons for NR1-KD, respectively; KS-test between NR1-KD and WT for P13/14 recovery curve $p<0.01$, and for P15/16, P17/18 recovery curve $p<0.001$. 

\[A\] P11/12

\[B\] P13/14

\[C\] P15/16

\[D\] P17/18
Preliminary density analysis of neurons in the immature (P11) MNTB shows possible high density of neurons in NR1-KD. Imaging of the presynaptic morphology was obtained through the anterograde lipid staining of the globular bushy cells using DiI crystals inserted into the aVCN of brains preserved in PFA. A z-stack composition of the images allowed for a 3-D reconstruction of the presynaptic terminals in the MNTB, which each wrap around a single MNTB principal neuron (example slice images A & B). Using the reconstruction we found there is preliminary evidence to suggest a larger number of calyces in the MNTB slices, as measured through the number of calyces imaged in a set window (C). mean ± SEM; n = 4 slices for WT and NR1-KD, each; student unpaired t-test, 2-tailed p-value *<0.05, **<0.01, ***<0.001; scale bar = 50 μm (A & B).