Non-stationary Analysis of Synaptic Transmission at a Central Synapse:
Comparing the quantal sizes used in evoked vs. spontaneous release

by

Zoltan Nagy

A thesis submitted in conformity with the requirements
for the degree of Master of Science,
Graduate Department of Physiology,
University of Toronto

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Dedicated to Dr. Lu-Yang Wang  
Without whom it would not have been

"The sciences do not try to explain, they hardly even try to interpret, they mainly make models. By a model is meant a mathematical construct which, with the addition of certain verbal interpretations, describes observed phenomena. The justification of such a mathematical construct is solely and precisely that it is expected to work."

- John von Neumann

"If our small minds, for some convenience, divide ... this universe into parts - physics, biology, geology, astronomy, psychology, and so on - remember that nature does not know it!"

- Richard P. Feynman
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ABSTRACT

The postsynaptic response to neurotransmitters released from a single synaptic vesicle is the smallest amount of possible signal transmission in the nervous system, called quantum. Release of synaptic vesicles can occur spontaneously or due to an action potential. One of the assumptions of the quantal hypothesis is that the quanta involved in evoked events are indistinguishable from those occurring spontaneously. We apply variance-mean analysis, desynchronized release in strontium (Sr²⁺), and unitary quantal release in cadmium (Cd²⁺) at single axosomatic glutamatergic synapses, to show that the average quantal size underlying evoked excitatory synaptic current (eEPSC) is significantly larger than those involved in spontaneous release (sEPSC). This increase in average size may indicate a preferential selection of certain quanta in evoked release out of all the quanta available. Our data may be important to refine the quantal theory on neurotransmitter release and the current view of synaptic transmission at mammalian central synapses.
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The members of the Wang lab, Tina Epps, Mike Fedchyshyn, Indu Joshi and Shahira Shokralla provided input on a daily basis. Although this input was often driving me crazy I can only mention them here as usually in theses there is only an "Acknowledgements" and no "Annoyments" section allowed. Maybe I should start...? Okay, just kidding....
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## Abbreviations

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<tr>
<td>Excitatory Post-synaptic Current</td>
<td>EPSC</td>
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<tr>
<td>Evoked miniature EPSC</td>
<td>eEPSC</td>
</tr>
<tr>
<td>Spontaneous miniature EPSC</td>
<td>sEPSC</td>
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<tr>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate</td>
<td>AMPA</td>
</tr>
<tr>
<td>Cadmium ion</td>
<td>Cd$^{2+}$</td>
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<tr>
<td>Calcium ion</td>
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<td>Extracellular Ca$^{2+}$ concentration</td>
<td>[Ca$^{2+}]_o$</td>
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<td>Intracellular Ca$^{2+}$ concentration</td>
<td>[Ca$^{2+}]_i$</td>
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<td>Medial Nucleus of the Trapezoid Body</td>
<td>MNTB</td>
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<tr>
<td>Number of release sites</td>
<td>N</td>
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<tr>
<td>N-methyl-D-aspartate</td>
<td>NMDA</td>
</tr>
<tr>
<td>Potassium ion</td>
<td>K$^+$</td>
</tr>
<tr>
<td>Probability of release</td>
<td>P$_r$</td>
</tr>
<tr>
<td>Quantal size</td>
<td>Q</td>
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<td>Strontium ion</td>
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<tr>
<td>Tetraethylammonium</td>
<td>TEA</td>
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<td>Variance – Mean Analysis</td>
<td>VMA</td>
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1 - Introduction

It is generally believed that processes such as learning and perception of all animals as well as the cognitive mind of humans are manifestations of brain (or nervous system) activity (Jessell et al., 1995). That is, all the voluntary actions exhibited by animals, along with perception, the life sustaining processes (such as heartbeat and breathing), and homeostatic mechanisms (e.g. temperature, water balance) are carried out by the specialized functions of the nervous system. Depending on the organism a nervous system may contain anywhere from a few to several 100 billion brain cells and it is believed to produce all of its features by communication among these individual units through interconnections, called synapses (Jessell et al., 1995).

Our studies specifically focus on the smallest unit of information transfer through synapses, called the quantal response. During these investigations we combine experimental and theoretical techniques. In the following three sections we shall review the relevant aspects of synaptic transmission, our experimental preparation as well as the theoretical model we applied. In the final section of this chapter we shall state our hypothesis and describe the details of our investigation.

1.1 Synaptic Transmission

Studying synaptic mechanisms is important because coding and transmission of information in the nervous system is achieved through the timing and pattern of the transmitted signals. Fluctuations in timing represent potential error or signal degradation. On the other hand, controlled modulations in transmission are thought to be responsible for learning and information processing (Bekkers and Stevens, 1990; Reid and Clements, 1999). Modulations of synaptic
transmission can be brought about by pre-synaptic and/or post-synaptic means, that is, by changing the characteristics of the transmitting cell and/or that of the cell receiving the information, respectively.

The nervous system consists of several distinct cell types. Nerve cells are called neurons, which are specialized for receiving, processing and transmitting signals. In order to achieve these tasks, a nerve cell has special morphological adaptations. Apart from the cell body, neurons may also have a dendritic tree, an axon and a presynaptic terminal (Fig. 1.1).

![Figure 1.1 Anatomy of a Neuron](image)

This schematic diagram describes the major anatomical parts of most neurons (not drawn to scale). The number of dendrites and the size of the dendritic tree vary among neurons. Also, the length of the axon, as well as the number of collaterals are variable.

Signal transfer between two neurons usually occurs at a specialized cellular structure, namely the synapse. The actual mechanism is a transduction whereby the intracellular electric signal of one
cell is converted to an extracellular chemical signal. This chemical signal reaches another cell only to be converted back again to an electrical signal which passes on as such intracellularly.

We will mainly be concerned with transmission through the synapse and not with how signal conduction occurs in the rest of the cell. Furthermore, we will review the aspects of synaptic transmission as it pertains to the Calyx of Held synapse in the auditory brainstem, the experimental preparation of this thesis.

1.1.1 Presynaptic Mechanisms

The main role of the presynaptic terminal is the transduction of an electrical signal to a chemical one. The actual mechanism is fairly involved. This complexity is advantageous however, in the sense that it allows for modulation at many different, but interrelated steps.

The general presynaptic mechanisms are similar, to a large extent, in all neurons. As the electrical signal, called the action potential, invades the presynaptic terminal, calcium ion influx occurs through Ca\(^{2+}\) channels, which open in response to the change in voltage (i.e. they are voltage-gated). Through some biochemical mechanism this transient rise in intracellular Ca\(^{2+}\) concentration, causes the neurotransmitter-containing vesicles to fuse with the cell membrane and in the process release their contents into the extracellular space, called the synaptic cleft (see next section).

There are several Ca\(^{2+}\) channel types. Ca\(^{2+}\) ions can enter the presynaptic terminal through selective, non-selective, voltage- and ligand-gated channels as well as through an energy dependent pump, the Na\(^{+}\) / Ca\(^{2+}\) exchanger (Hille, 1992; Meir et al., 1999). Of all these routes we
will only be concerned with those that respond to electrical stimulation of nerve cells even though all the other types add other mechanisms to modulate synaptic transmission and thus offer further pathways to learning and information processing. It should also be mentioned that Ca$^{2+}$ can also be released from intracellular stores through IP$_3$ and ryanodine type channels and this mechanism has been implicated in the modulation of quantal properties at some synapses (Emptage et al., 2001; Llano et al., 2000).

Even after narrowing down our focus to voltage gated channels only, there are several different channels still to consider. These differences manifest themselves in the rate at which the channel lets through Ca$^{2+}$ and also in the amount of time the channel takes before it closes again once it opened due to a voltage signal. These two characteristics of Ca$^{2+}$ channels form an available stage for synaptic modulation. This is because Ca$^{2+}$ is an important intracellular signaling ion for synaptic transmission. Modulating the Ca$^{2+}$ availability within the synaptic terminal leads to controlled and well-defined changes in the release of synaptic vesicles.

Vesicular release does not always occur in response to a presynaptic action potential. This is because release events are probabilistic. That is when a signal invades the presynaptic terminal, there is a distinct probability of whether an available vesicle will fuse with the membrane and release its contents or not. This probability is usually called the probability of release (often denoted as P$_r$). Intracellular calcium ion concentration ([$Ca^{2+}$]$_i$) is directly related to the probability of release.

From the work of Bernard Katz and Ricardo Miledi it has been known since the 1960's that Ca$^{2+}$ is responsible for vesicle release (Katz and Miledi, 1967). However, the molecular mechanisms
which link the transient rise in \([\text{Ca}^{2+}]_i\) to the release of a vesicle are complicated and to the most part still unclear. One thing is evident – in order for the \([\text{Ca}^{2+}]_i\) to be an important factor in vesicle release, its level must be monitored and this is done by molecular \(\text{Ca}^{2+}\) sensors in the terminal. Currently, there is increasing evidence that the most likely candidate for being a \(\text{Ca}^{2+}\) sensor is Synaptotagmin (Fernandez-Chacon et al., 2001; Geppert et al., 1994). This protein resides in the membrane of synaptic vesicles and in response to \(\text{Ca}^{2+}\) binds with several other proteins, collectively known as the Docking (SNARE) Complex, to bring about fusion of the vesicle with the cell membrane and subsequently exocytosis.

Calcium ions have other roles in presynaptic vesicle release. For example releasing the vesicles from the cytoskeleton and thus making them available for fusing with the cell membrane is known to be a \(\text{Ca}^{2+}\) dependent step. More importantly however, it has been proposed that \([\text{Ca}^{2+}]_i\) is related to the extent to which a vesicle fuses with the cell membrane. Initially, it was thought that vesicles completely fused with the membrane (often called a collapse) and emptied their contents. In the process the membrane of the vesicle would be incorporated into the cell membrane itself. Subsequently, this extra membrane area would need to be removed by endocytosis to prevent the continual growth of the surface area of the terminal. Recently however it was shown that a full collapse is not the sole method of vesicle release (Alvarez et al., 1993) and there may be partial release events, called "Kiss and run" events (Stevens and Williams, 2000). In fact it seems wasteful that full collapse should occur at all (Neher, 1993).

Nonetheless, kiss-and-run events have been shown to correlate with \(\text{Ca}^{2+}\) dynamics. In one study, for example, raising the extracellular \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_o\)) was directly related to a shift of exocytosis to the kiss-and-run mode (Ales et al., 1999). Another investigation showed that increasing \([\text{Ca}^{2+}]_i\) inhibits, and at high enough levels abolishes endocytosis of synaptic vesicles.
in a negative feedback fashion. (von Gersdorff and Matthews, 1994). Furthermore, it has been shown that repeated stimulation causes an increase in Ca\textsuperscript{2+} influx. Coupling these two latter findings together leads to an exocytosis mechanism which is regulated in an activity dependent manner.

These mechanisms introduce further possibilities for the modulation of transmitter release. For our purposes the extent of synaptic vesicle fusion is important because we intend to investigate the postsynaptic response to the amount of neurotransmitters released. The possibility of an activity dependent mechanism, regulating the amount of neurotransmitter released, allows us to speculate that the preferential adjustment of release events may occur.

For our hypothesis the proteins in the Docking Complex form an important step in synaptic transmission as they may assemble in different combinations for spontaneous and evoked release events. Although our methods of investigation do not allow for validating this difference, the possibility allows us to hypothesize.

In one of our experimental protocols we shall replace the extracellular Ca\textsuperscript{2+} with Sr\textsuperscript{2+}. Strontium ions permeate voltage-gated Ca\textsuperscript{2+} channels and Pr is related to the intracellular levels of Sr\textsuperscript{2+} in a similar fashion to Ca\textsuperscript{2+}. However the intracellular dynamics of [Ca\textsuperscript{2+}] and [Sr\textsuperscript{2+}] are different. Strontium ions are removed more slowly and there is also a difference in the binding to sensors (Xu-Friedman and Regehr, 2000). As a result, using Sr\textsuperscript{2+} in place of Ca\textsuperscript{2+} results in a desynchronized and delayed release of synaptic vesicles.
1.1.2 The Synaptic Cleft

The synaptic cleft is the specialized extracellular space between the two cells forming the synapse. It is specialized in the sense that the distance between the cells here is larger (≈ 200-300Å) than the normal extracellular space between adjacent cells. Another distinction of the extracellular space of the synaptic cleft is the large amount of carbohydrates found. The pre- and postsynaptic membranes contain glycoproteins, which are presumably important in synapse formation and maintenance. The large carbohydrate content of the cleft is likely due to these proteins (Levitan and Kaczmarek, 1997).

The neurotransmitters, released from the vesicles of the presynaptic terminal, travel across the cleft by passive diffusion. Once this chemical signal reaches the postsynaptic cell the neurotransmitters bind to specific receptor proteins in the postsynaptic membrane, as will be discussed in the next section. The leftover neurotransmitters are quickly cleared from the cleft to reduce the chances of erroneous signals being transmitted which would not be associated with a presynaptic signal. There are several methods by which neurotransmitters are removed from the cleft. They can be taken back up by the releasing cells, perhaps after some chemical modifications, just to be stored again in vesicles and re-used for release. Also, there may be enzymes present in the cleft, which incapacitate the transmitters by a chemical conversion. These enzymes have kinetic rates slow enough for allowing diffusion to the adjacent cell for a large portion of the transmitters, but fast enough to inactivate the surplus transmitters. Finally, the leftover transmitters may be taken up by adjacent supporting, non-neuronal brain cells, called astrocytes. At the neuromuscular junction enzymatic removal is the favoured method, while at central synapses reuptake by the releasing cell and astrocytes are more prominent (Jessell et al., 1995).
Whichever the mechanism(s) used at a specific synapse, however, the removal of the neurotransmitter is a determinant factor of the time course of the transmitter in the cleft. Although it is extremely hard to experimentally measure this exact time course, some attempts have been made at the neuromuscular junction and at cultured hippocampal neurons (Clements et al., 1992; Katz and Miledi, 1973).

1.1.3 Postsynaptic Mechanism

As the presynaptic terminal, the postsynaptic density also specializes in transduction. In this case the signal is converted from chemical back to electrical. Again the actual mechanism is quite complex and it offers many steps where regulation may and does occur. As with the presynaptic mechanisms, the postsynaptic machinery bears similarities across different nerve cell types.

The area of the postsynaptic cell, directly opposite to the presynaptic terminal, is specialized for transducing the chemical signal back to an electrical one. After traveling the dimensions of the synaptic cleft the neurotransmitter molecules bind to postsynaptic receptors. A receptor may be ionotrophic or metabotropic (Jessell et al., 1995). Our focus will be on ionotrophic receptors as these are involved with the transmission at the synapse where we have done all of our experiments. The different types of ionotrophic receptors are able to produce either inhibitory or excitatory responses. We will only concentrate on the latter type because the synapse we study belongs to this group. The amino acid, Glutamate, is responsible for most of the excitatory neurotransmission in the brain including the Calyx. Its two main postsynaptic receptors are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate) and NMDA (N-methyl-D-aspartate) receptors (Scannevin and Huganir, 2000).
The protein machinery of the postsynaptic cell, which is involved with signal transmission, is complex and complicated as in the case of the presynaptic terminal, but for the purposes our program here it is not necessary to explain in detail. The only important feature to point out is that these proteins, collectively called the Postsynaptic Density, allow for the regulation of transmission (Kennedy, 2000).

1.2 Quantal Hypothesis

The ultimate aim of Neuroscience is to elucidate how the nervous system accomplishes its tasks. Generally scientific disciplines advance in steps when the current knowledge of a given field is collected and fitted into a conceptual construct, called a model. To elucidate the functions and mechanisms of the nervous system neuroscience also relies on these models. A given model may or may not turn out to be correct in the long run, but at the time of its creation it summarizes the current understanding and it does not contradict the postulates that were required for fitting it into the current framework of knowledge. This is the case also with the Quantal Hypothesis, which is a model of synaptic transmission, proposed by Katz and del Castillo in the early part of the 1950's (del Castillo and Katz, 1954a;del Castillo and Katz, 1954b).

Two facts that we already mentioned above are important for understanding the Quantal Hypothesis. One is that the neurotransmitters are stored in vesicles. The postsynaptic stimulus due to the contents of one vesicle is called a quantal response or a Quantum. The other crucial component of the Quantal Hypothesis is that the [Ca^{2+}]_i is related to the probability of release, or \( P_r \). Because if there are more than one vesicle available for release in a given presynaptic terminal then the increase in \( P_r \) makes the simultaneous co-release of several vesicles possible (i.e. probable). Therefore, the message of the Quantal Hypothesis is that any amount of synaptic transmission will have to be an integer multiple of quantal responses.
Individual quanta have were first observed by Fatt & Katz (1952) at the neuromuscular junction. They noticed that even in the absence of presynaptic stimulation, small events — miniature events as they called them — occurred spontaneously (Fatt and Katz, 1952). These events seemed to have a certain, relatively constant amplitude, much smaller than those observed following a presynaptic action potential, and the exact cause of it wasn’t clear — especially because they occurred in a random manner. However, the time course (i.e. the rise-time and the decay time constant — see Fig 1.2) of these miniatures was similar to the evoked ones. In fact if these small, random miniature events could be synchronized in some way then they could give rise to the much larger evoked events. This idea was further supported by the observation that evoked postsynaptic events did not have a constant amplitude but displayed a fluctuation on consecutive stimulations. Based on these observations and facts del Castillo and Katz formulated their model.

![Stimulation artifact](image)

**Figure 1.2 Sample traces and the terminology of an EPSCs**
The basic underlying principles of the Quantal Hypothesis rely on a Binomial Probability Distribution, the general ideas of which can be found in every introductory text on probability and statistics (see for example (Rosner, 2000)). Let $P_r$ be the probability of release at a presynaptic terminal and $N$ the number of vesicles available for release in response to an action potential. Then on a given stimulation the number of vesicles released will vary probabilistically with a mean response equal to $NP$, according to the probability model used.

The Quantal Hypothesis is an elegant idea. But as so often in science, this model is probably a simplified version of the true nature of synaptic transmission – a first approximation. In particular the originally used statistical treatment seems to be an overly rigid framework that has led to quite a bit of controversy. First of all, the Binomial Probability Model, which Katz and del Castillo used, assume repeatable trials. That is, the number of release sites and the probability of release should be constant from trial to trial. Secondly, the probability of release should be constant for all vesicles. Some authors, as late as 1977, went as far as questioning the meaningfulness of transmitter release statistics (Barton and Cohen, 1977). Others tried to include these possible variations in the $N$ and the $P_r$ on consecutive stimulations as well as the distribution of the magnitude of $P_r$ among the release site on any given stimulation (McLachlan, 1978; Quastel, 1997; Uteshev et al, 2000).

At this point we conclude the following. The known mathematical functions may not be capable of describing the transmission or if they are capable then they may not be practical. Nonetheless the general idea of the synchronized quanta constituting evoked responses, seems generally accepted and uncontested, not only at the neuromuscular junction but also within the CNS (Wall and Usowicz, 1998). We also believe this to be true and intend to probe deeper into the model.
without relying solely on a binomial probability model. Even in their original paper del Castillo and Katz pointed out an important fact:

A large evoked response "... is made up of units of the same size (though not necessarily composed of the same individuals) as the spontaneous miniature..." events.

To determine whether in a central synapse the quanta composing the evoked events (eEPSC) are identical in amplitude to those responsible for spontaneous miniatures (sEPSC) is our thesis. We will apply both theoretical and experimental methods to make this comparison.

1.3 Our preparation and it's advantages

As stated above, the Quantal Hypothesis and all of its assumptions are based on experiments performed at the neuromuscular junction. It is however controversial if this theory applies to central synapses in the mammalian brain, and if it does it is unclear to what extent a direct application is possible. This is due to the fact that structures similar to the neuromuscular junction are not usually found in the central nervous system. Although central neurons receive a large number of inputs, these inputs originate from several distinct cells, which are possibly located in different areas. Therefore selective stimulation of presynaptic inputs is difficult. It is also not feasible to associate sEPSCs with a given input. Furthermore, input to central neurons most often occurs through dendrites which, for the purposes of electronic recording at the cell body, introduces distortions due to cable filtering (Johnston and Wu, 1995).

The auditory pathway, as all the other sensory modalities, displays a number of specific organs and cellular structures, which allow it to reliably receive, transmit and process the kind of information for which it was specialized. One of these specializations is the presynaptic terminal under investigation in this study, called the Calyx of Held. This structure is morphologically
similar to the neuromuscular junction and thus allows for an analogous study within the central nervous system.

1.3.1 A Brief Overview of the Auditory Pathway*

The hair cells of the cochlea provide input to the dendrites of the Spiral Ganglion neurons. The axons of these neurons form the Auditory Nerve. The fibers of this nerve project to the ipsilateral Cochlear Nucleus in the brain stem. The Cochlear Nucleus processes the information it receives from the Auditory Nerve and subsequently distributes it to higher auditory nuclei. One of the three major output pathways from the Cochlear Nucleus is to the Superior Olivary Complex. The Superior Olivary Complex is located ventrally in the brain stem and it receives inputs from both the Ipsilateral and Contralateral Cochlear Nucleus. Hence it is the first stage where convergence of information from both ears takes place. The Superior Olivary Complex consists of three distinct nuclei, one of which, the Medial Nucleus of the Trapezoid Body (MNTB), contains the synapses on which we will focus.

The properties of sound stimulus received by the cochlea are encoded by several mechanisms. First a tonotopic arrangement of cells in the auditory centers ensures a more reliable transmission of signals by different cells in the pathway adapting well for a smaller bandwidth. This tonotopic arrangement in the MNTB results in a pattern where the higher the frequency response of a given cell the more medially is it located. Secondly, sound stimulus characteristics are encoded by the pattern and timing of action potentials generated in neurons of the auditory pathway and it is also thought that localization of the sound source is achieved by computing the temporal differences between the signals received by either cochlea (Releigh, 1907). Hence it is of paramount
importance that the quality of the signal be preserved as it passes from one level to another in the auditory pathway.

The auditory nerves preserve the signal, at least at low frequencies, by a method called phase-locking which means that the axons in the nerves propagate action potentials in response to a specific phase of the cyclic signal (Trussell, 1997). Different regions of the Cochlear Nucleus contain different cell types. One cell type, the Globular Bushy Cells, reside in the Ventral Cochlear Nucleus. Several axons of the Auditory Nerve terminate presynaptically on a given globular bushy cell. As a result of this convergence the action potential pattern and timing of globular bushy cells are better than those of the auditory nerves (Joris et al., 1994). That is, the phase locking to the original signal is better. The output of the globular bushy cells terminates on neurons of the contralateral MNTB. At this stage, the ever-present necessity for signal preservation results in the specialized structure, called the Calyx of Held, which is the presynaptic terminal of a Globular Bushy Cell.

Input to MNTB neurons is both inhibitory and excitatory and originates from several areas in the brain stem. The major input, which originates from the contralateral Cochlear Nucleus, is excitatory. The neurotransmitter is glutamate. There are several different cell types in the Cochlear Nucleus. The major input is from the Globular Bushy Cells. The other cell types may also form synapses with cells in the MNTB in an axo-dendritic manner. Inhibitory input to the MNTB originates predominantly from ipsilateral nuclei which surround the Superior Olivary Complex and are collectively called the Periolivary Nuclei. The neurotransmitters are γ-

* Material in this review is contributed by too many authors to individually acknowledge. Unless otherwise indicated please see the following review for original sources (Ehret and Romand, 1997)
aminobutyric acid (GABA) and Glycine and the synapses formed, as characteristic for inhibitory inputs, are perisomatic (i.e. close to the soma rather than on dendrites).

The output of MNTB cells is solely inhibitory (Glycinergic) and it is directed to several other brain stem nuclei. Ipsilaterally, the main targets are the Periolivary Nuclei and the other two nuclei of the Superior Olivary Complex. Minor output pathways also point to both the ipsilateral and the contralateral Cochlear Nucleus, as well as, by way of colaterals, to neighbouring ipsilateral MNTB neurons. Since the input of the MNTB cells is excitatory and one-to-one from the Contralateral Cochlear Nucleus, the ultimate affect of MNTB output is inhibition due to sounds stimulus to the contralateral ear.

1.3.2 The Calyx of Held

As mentioned previously, the Calyx of Held is a unique structure which results from the necessity for signal preservation in the auditory pathway. This specialization is not only a favourable adaptation for perception but it also provides and excellent opportunity for experimental investigation of synaptic transmission.

The first important characteristic of this specialization is that every MNTB cell receives input from a single Globular Bushy Cell. Therefore, at this stage of the auditory pathway there is a one-to-one transmission. The second aspect of this morphological specialization is that the Calyx of Held provides input directly to the soma of the cells in the MNTB. This arrangement allows for a more reliable transfer of information both temporally and also ensuring high fidelity (i.e. high probability that if the presynaptic cell fires then so will the postsynaptic). The final aspect that we consider here is the shear size of the calyx. It is estimated to cover about 60% of the
surface of the postsynaptic cell body. This surface supports many sites where synaptic transmission can occur. Hence as an action potential invades the presynaptic terminal a large number of synaptic vesicles are released resulting in a single, large Excitatory Post-Synaptic Current (EPSC).

These three favourable structural specializations provide experimental as well as analytical advantages. The one-to-one correspondence allows us the convenience of not having to deal with converging inputs. That is if the inhibitory minor inputs are pharmacologically eliminated then if an EPSC is recorded from a cell in the MNTB then it responded to a single input. Experimentally the general procedure is to artificially stimulate the axons of the presynaptic cell, while recording the response from the postsynaptic cell. If there are converging inputs to a cell then it is fairly hard, if not impossible, to single out the input in question and only stimulate that one. For this preparation it is not an issue as there is only a single major input.

The axo-somatic synaptic arrangement allows for recordings to be made with good temporal resolution and it eliminates the cable filtering effects which are generally a problem when recording from a cell which has inputs to dendrites.

Finally, the large number of vesicles released in response to an action potential grant two experimental advantages. One is that the EPSC size is much larger than that of the background noise. Secondly, since presynaptic vesicle release is thought to be a probabilistic phenomenon, having many vesicles released per action potential reduces the number of times that the experiment needs to be carried out. As an analogy consider that a person aims to find out whether a certain type of coin is fair. He could either flip one of those coins 1000 times or
collectively 200 identical coins five times. The exact number of vesicle release sites within the calyx are not known. There are several estimates made with different methods and these estimated values range from 700 (Schneggenburger et al., 1999) to as high as 5000 vesicles (Sun and Wu, 2001).

There are several developmental changes concerning the physiology of the calyx, which relate to synaptic transmission. These changes cannot be ignored. One example is the fact that the different Ca\(^{2+}\) channels have different kinetic properties. These properties determine the amount of Ca\(^{2+}\) influx in response to an action potential and hence in turn would determine the probability of release. However, it seems that developmentally there is a predominant presence of P/Q – type calcium channels after the ages of 10 postnatal days (Iwasaki and Takahashi, 1998) and this removes the complications that we would otherwise encounter if the number of the different types of channels were constantly changing in an unknown manner. A second example concerns the postsynaptic receptors. We mentioned that Glutamate is specific to both AMPA and NMDA type receptors. Again these two receptors have different kinetic properties, which, in this case, determine the postsynaptic response characteristics. During EPSCs with contributions from both types of receptors, the early phase is due to the fast AMPA type receptors. On the other hand NMDA receptors take part in the later parts of the EPSC for two reasons. First, NMDA receptors have slower kinetics than AMPA receptors but more importantly there is a voltage dependent block of NMDA receptors by Mg\(^{2+}\) from the extracellular side (MacDonald and Nowak, 1990;Nowak et al., 1984). Therefore the NMDA receptors only open after the cell membrane has been sufficiently depolarized by the current through the AMPA receptors. Again it is important to know to what degree the different receptor types contribute to the postsynaptic response that we measure. As it turns out however, there is a preference for AMPA receptors in
synapses of the MNTB after the thirteenth postnatal day (Joshi and Wang, 2000). This
developmental down-regulation of NMDA receptors at the MNTB may be a result of the onset of
hearing, which in mice occurs at the postnatal age of 10-12 days (Futai et al., 2001).

There are also activity dependent changes in synaptic transmission. That is high-frequency
stimulus can modulate one or more steps of the transmission mechanism. For example it has
been observed that high-frequency stimulation has positive presynaptic effects in the form of
speeding up the process of replacing the released vesicles within the terminal (Wang and
Kaczmarek, 1998). As another example the $[\text{Ca}^{2+}]_i$ after consecutive high-frequency stimuli is
found to be variable (Borst and Sakmann, 1998). There are many other affects, such as Post-
Tetanic Potentiation/Depression and Long Term Potentiation/Depression at many central
synapses but these modulations have not been thoroughly studies at the Calyx of Held.

To remove the potential confounding factors of these variables we used mice older than the 13th
postnatal day and our protocols included only single stimuli.

1.4 Variance-Mean Analysis

When the Quantal Hypothesis was introduced above we mentioned that the originally assumed
binomial model proved to be an overly rigid framework to describe all the capacities of synaptic
transmission. There we also pointed out that there have been many attempts to expand on this
model to be able to explain a larger set of the observed phenomena. One of these attempts is a
recently developed method called Variance-Mean Analysis (VMA) (Clements and Silver, 2000).
The main advantage of this new method is that it does not rely on the assumption that the
synaptic properties (e.g. $P_t$) are uniform across the release sites of a given synapse.
Variance-Mean Analysis is basically still a binomial model with a few reasonable additions, which allow a broader range of application and prediction. Mathematically, it can be derived as follows. We can start with a general equation (Bekkers and Stevens, 1990)

\[ s^2 = a^2 Np(1 - p) + a^2 Npc_m^2 \]  

(Equation 1.1)

where \( s^2 \) is the standard deviation of the EPSC, \( a \) is the average quantal size, \( p \) is the average release probability and \( N \) is the number of vesicle release sites. The second term (i.e. \( a^2 Npc_m^2 \)) is the variance introduced by the site to site variations and \( c_m \) is the coefficient of variation of the miniatures. The coefficient of variation is the standard deviation divided by the mean. For the binomial probability distribution the variance and the mean are:

\[ s^2 = Np(1 - p) \quad \text{and} \quad x = Np \]  

(Equation 1.2)

where \( x \) is the mean. Therefore the square of the coefficient of variation for the binomial distribution can be calculated as:

\[ c_s^2 = \left( \frac{s}{x} \right)^2 = \frac{s^2}{x^2} = \frac{Np(1 - p)}{(Np)^2} = \frac{1 - p}{Np} \]  

(Equation 1.3)

On the other hand the coefficient of variations for EPSCs using equation 1 is:

\[ c_e^2 = \frac{1 + c_m^2 - p}{Np} \]  

(Equation 1.4)

It is easy to see that if the site to site variations are small (i.e. \( c_m^2 \to 0 \)) then the coefficient of variation of evoked responses can be approximated by the binomial distribution (i.e. \( c_e^2 \equiv c_s^2 \)).

Now starting from equation 1.1 we can derive the equation used for the VMA:

\[ s^2 = a^2 Np(1 - p) + a^2 Npc_m^2 = a^2 \left[ Np - Np^2 + Npc_m^2 \right] = a^2 \left[ Np \left( 1 + c_m^2 \right) - Np^2 \right] \]  

(Equation 1.5)
Next we multiply the last term by the factor \( \frac{N}{N} \), which is really simply multiplying by the number one. This gives:

\[
s^2 = a^2 \left[ Np \left( 1 + c_n^2 \right) - Np^2 \right] = a^2 \left[ Np \left( 1 + c_n^2 \right) - \left( \frac{N}{N} \right) Np^2 \right] = a^2 \left[ Np \left( 1 + c_n^2 \right) - \frac{N^2 p^2}{N} \right] \quad \text{(Equation 1.6)}
\]

However, we know from equation 1.2 that the mean of the binomial distribution is given by \( Np \). Thus finally we may write:

\[
s^2 = a^2 \left[ x \left( 1 + c_n^2 \right) - x^2 \right] \quad \text{(Equation 1.7)}
\]

Within the square brackets in equation (7) we have the simple description of a parabola.

Therefore, if the binomial probability model is applicable (i.e. when \( c_n^2 \to 0 \)) then the variance and mean of EPSCs are related to each other by a parabola.

Unfortunately, of the many publications dealing with this topic, all use somewhat different notation. To avoid confusion, the form of the equations we shall be using from now on is given in (Clements and Silver, 2000) and it is as follows:

\[
y = Ax - Bx^2 \quad \text{(Equation 1.8)}
\]

Here 'y' represents the variance and 'x' again is the mean of EPSCs with

\[
A = Q_w \left( 1 + c_n^2 \right) \quad \text{and} \quad B = \frac{1}{N} \quad \text{(Equation 1.9)}
\]

From now on we shall use \( Q \) to represent the quantal size instead of 'a' in equation (1). The subscript 'w' represents the fact that \( Q \) is the weighted average of the quantal sizes.
For our purposes the most important aspect of the parabolic relation of equation (7) or (8) is the fact that the initial slope of the rising phase of the parabola is related to $Q$. To see this we differentiate equation (8)

$$\frac{d}{dx} y = A - 2Bx \quad \text{(Equation 1.10)}$$

Evaluation of this expression at $x = 0$ gives the slope of the tangent line to the initial, rising phase of the parabola. Thus:

$$\frac{d}{dx} y(0) = A - 2B(0) = A = Q_w (1 + c_w^2) \quad \text{(Equation 1.11)}$$

Simply put, the coefficient of the $x$-term in the equation of the parabola is related to the quantal size in a simple fashion.

To make use of this theoretical model in practice, the experimental protocol would be as follows. By now we know that the amplitude of an EPSC is related to the $[\text{Ca}^{2+}]_i$. To see it clearly suppose we have 100 vesicles for release. The higher the probability of release the larger the number of vesicles that will be released on any given presynaptic stimulus. Since the $[\text{Ca}^{2+}]_i$ is directly related to $P_r$, manually adjusting the calcium ion influx levels enables one to set the mean amplitude of the EPSCs. To set the $[\text{Ca}^{2+}]_i$ levels, one may use two different $\text{Ca}^{2+}$ channel blockers. Cadmium ions ($\text{Cd}^{2+}$) non-selectively block $\text{Ca}^{2+}$ channels from the extracellular side. Introducing $\text{Cd}^{2+}$ to the bathing solution limits the amount of $\text{Ca}^{2+}$ influx when the channels open in response to a presynaptic action potential. On the other hand tetraethyl-ammonium (TEA) selectively blocks the high-threshold, voltage gated $\text{K}^+$ channels. The efflux of potassium ions is important in the repolarizing phase of an action potential (see for example (Sherwood, 1993)). Hence blocking $\text{K}^+$ channels lengthens the presynaptic action potential by broadening the spike width without changing other properties (Wang and Kaczmarek, 1998). As a result, voltage gated
Ca\textsuperscript{2+} channels remain open for a longer time and consequently allow more Ca\textsuperscript{2+} into the terminal. Using these two channel blockers the release probability can be set (and hence the EPSC amplitudes) freely and accurately.

At each probability setting one would measure the mean and the variance of the EPSCs and plot them against each other on a scatter plot. With the subsequent fitting of a parabola to these points allows for the determination of the coefficient 'A' in equation (8). If then one is able to measure or estimate the value of \( c_n \), then the value of the quantal response can be calculated from equation (11), namely

\[
Q_w = \frac{A}{1 + c_n} \quad \text{(Equation 1.12)}
\]

We estimated the value of \( c_n \) from that of the coefficient of variation for the sEPSCs.

One of the important conclusions of Variance – Mean Analysis is as follows. If the release probabilities are not uniform among the release sites then at high probability settings (i.e. \( P_r \to 1 \)) the plot will deviate from that of a parabola. Therefore, if we observe a good parabolic fit in the above procedure then it is a good indication of the fact that the site-to-site variation in \( P_r \) is small.

**1.5 Hypothesis**

Our aim with this project was to compare the quantal sizes of sEPSCs and eEPSCs in a central synapse. Even the founders of quantal hypothesis concluded that the evoked responses might not contain the exact same quanta as would be observed when recording sEPSCs (del Castillo and Katz, 1954a). Although at the neuromuscular junction the average sizes seem to be similar, there is an increasing body of work, from both the neuromuscular junction and the CNS, indicating a
difference in several aspects between these two types of quanta. Furthermore there are some other results which suggest that the quantal characteristics of transmission in the CNS may differ from those at the neuromuscular junction. Some of these are:

-- Bekkers and Clements used Strontium ions to desynchronize the quantal content of evoked events in rat dentate granule neurons. They observed that the desynchronized quanta had an average size significantly larger than that of the sEPSCs (Bekkers and Clements, 1999).

-- Relatively recently it has been shown that complete emptying of vesicles upon fusion with the cell membrane is not the only mechanism of release. Partial opening and release, termed “kiss-and-run”, is also observed (Ales et al., 1999; Alvarez et al., 1993; Stevens and Williams, 2000).

-- It has also been observed at the crayfish neuromuscular junction that there are different docking complexes for sEPSCs from those of eEPSCs (Hua et al., 1998).

-- While the at the neuromuscular junction the distribution of sEPSC amplitudes can be described by a single Gaussian curve, within the CNS there is a significant portion of sEPSCs whose amplitudes are much larger then would be predicted (Auger and Marty, 2000).

-- Finally, within the neuromuscular junction of mice in the absence of presynaptic release the postsynaptic receptors are quickly removed from and disassembled (Akaaboune et al., 1999). This may suggest that sEPSCs serve a purpose of maintenance while eEPSCs are used for transmission.

Based on these findings of other investigators we intend to compare the quantal sizes of spontaneous events with those of evoked ones. All our experiments include non-stationary methods meaning that we observe the quantal content of evoked events directly, during a stimulation, rather then estimate by indirect means, from time-invariant situations.
We hypothesize that sEPSCs represent a different set of available quanta from those that combine to form a full-scale EPSC event and that they would have dissimilar average sizes. Whether the difference arises from a graded level of fusion, a unique fusion protein machinery, differences in vesicle sizes, variations in neurotransmitter concentration among the vesicles, a different rates at which the neurotransmitters are removed from the cleft or from the number of postsynaptic receptors is not clear at the present time and our methods do not allow for the investigations of these variables. However, the exploration of these factors provide incentives for future investigations into the precise mechanism of synaptic transmission.

This project is novel and to our knowledge has not been done to this extent in the Calyx of Held – MNTB synapse.
2 - Methods

In order to carry out our program of probing the quantal nature of synaptic transmission in the central nervous system and to test our hypothesis, we made electrophysiological recordings from mouse brain stem slices. The data collected was then analyzed using commercially available software on a PC under the Windows operating system.

2.1 Experiments

A given experiment consists of two separate stages. The first an animal is dissected and the brain stem slices are prepared. The slices are then used for the electrophysiological experiments. These two steps will be described in this section. The methods employed here have previously been described (Forsythe, 1994; Takahashi et al., 1996).

2.1.1 Brain Stem Slice Preparation

In these experiments we used CD1xCD57 or CD1x129SV/EMS mice between the ages of postnatal days 13-20. After decapitation (in accordance with the guidelines set out by the Laboratory Animal Services Committee of the Hospital for Sick Children), we rapidly removed the brain and submerged it in an ice-cold bicarbonate-buffered artificial cerebrospinal fluid containing in mM NaCl 125, KCl 2.5, NaHCO₃ 26, NaH₂PO₄ 1.25, Na-pyruvate 2, myo-inositol 3, glucose 10, CaCl₂ 1.5, MgCl₂ 1. The pH of this solution was 7.4 and the osmolarity was 300 miliosmols / liter. This fluid was saturated with 95% O₂ and 5% CO₂ by directly bubbling the gasses into it. After removing the forebrain the spinal cord the brain stem was glued onto a vibrotome stage (Leica VT1000S) and the region that contains the MNTB was cut into 4-6 transverse slices of 250 µm thickness. These slices were then incubated (Isotemp 210, Fisher
Scientific) for 1 hour at 37 °C. Following the incubation the slices were allowed to equilibrate with the ambient room temperature (20-22 °C) for at least 10-15 minutes before experiments were performed on them. After this time the slices were transferred into a recording chamber, which was located under the microscope (Olympus Plan 4x/0.1w). While in the recording chamber, the slices were stabilized by a U-shaped, platinum clip, for which the two parallel sections were strung with 3-4 nylon threads. This allowed easy access to the cells within the MNTB while the tissue was appropriately immobilized. The chamber was continually perfused with the same artificial cerebrospinal fluid that was used for the dissection, with the addition of two postsynaptic receptor blockers. Bicuculline (GABAa receptor blocker) and Strychinine (Glycine receptor blocker). Both of these pharmaceuticals block inhibitory transmission. For our experiments bath-exchange perfusion systems are not adequate for two reasons. First of all they tend to be too slow for purposes of exchanging the bathing solution in experiments when we need to change the composition of the artificial cerebrospinal fluid. When using the bath exchange perfusion system where one exchanges the bathing solution in the chamber by mixing in a new solution with that already present and waits for the complete replacement of the old solution. This process takes a long time and also extent of exchange is hard to determine. The other problem with bath-exchange perfusion is that it non-linear process. That is, in different areas of the chamber the two solutions exchange at different rates and therefore steady state is hard to achieve. For these reasons, we used a so-called local, fast perfusion system (Fig 2.1), designed by Dr. Lu-Yang Wang, in which a rectangular perfusion barrel (56-800S, Warner) is placed onto the slice and adjacent to the MNTB (Fig 2.1). In this arrangement, the stream of solution exiting the barrel actually has a linear range of about 2 mm and covers the entire area of the MNTB, and as a result, introducing a new solution is fast and complete. As mentioned earlier the MNTB is innervated by the contralateral cochlear nucleus. These axon bundles run parallel to
each other as they cross the midline. Therefore all the axons, targeting neurons of the MNTB, can be stimulated at once. We used a bipolar stimulating method where the slice is actually clipped between the two prongs of the stimulating electrode. The electrode is positioned perpendicular to the direction of the axons and between the midline of the slice and the MNTB (Fig 2.1).
2.1.2 Electrophysiological Recordings

For the electrophysiological recordings we used Kwick-Fil electrodes (MTW 150F-4, World Precision Industries) which we pulled with the help of PP-830 Narashige machine. The electrodes were filled with an intracellular solution containing in mM K-gluconate 97.5, CsCl 32.5, EGTA 5, Hepes 10, MgCl_2 1, lidocaine N-ethyl bromide (QX314) 3 and TEA 30 mM. The pH of the intracellular solution was 7.2. The intrinsic resistance of the recording electrodes was 2-4 MΩ. The recording electrodes were guided to the cells by electronic manipulators (SMI controller, Mini 23 arms made by Luigs & Neumann and CV-203BU headstage made by Axon Instruments, Foster City, CA) and followed visually on a monitor (VM-902U, Hitachi) under a submersion lens (LUMPlanFL 60x/0.9w, Olympus). Until the electrode was positioned on a cell the intracellular solution was under positive pressure to force a stream of the solution. This helps to avoid mixing of the intracellular solution within the pipette and the bathing solution, and keeps the tip of the electrode clean of tissue and other debris. Once a cell of the MNTB was touched by the recording electrode, the positive pressure was removed from the recording electrode and we waited for a GΩ seal to form before the cell membrane area under the tip of the electrode was broken through by suction or a high frequency electrical pulse. Upon breakthrough the threshold of stimulation was determined. That is the stimulation intensity was increased until EPSCs were evoked in an all-or-none fashion. For all the experiments the stimulation intensity was set to 20-30% higher than this threshold. The experiments consisted of whole-cell, voltage-clamp recordings made from visually identified MNTB neurons. The signals were amplified by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). After breakthrough the series resistance of the whole-cell configuration was 4-8 MΩ. For the full-scale EPSC recordings this series resistance was compensated by 90% with a lag time of 10-15 μs and the data was digitized at 20 kHz and low pass filtered at 2 kHz. For sEPSCs as well as the eEPSC recordings
in Sr$^{2+}$ and Cd$^{2+}$ the compensation was off, and the data was digitized at 50 kHz and low pass filtered at 5 kHz. All the data was acquired on-line with pClamp7 software (Axon Instruments, Foster City, CA) using a Pentium II pro computer with 64 MB RAM and the Windows 95 operating system.

Figure 2.2 Schematic diagram of the Calyx of Held preparation

2.2 Experimental protocols

Applying several methods we attempted to compare the sizes of quantal events responsible for spontaneous and evoked transmission. These methods are VMA, desynchronized release in strontium and unitary quantal release in high concentration of cadmium. In order to carry out the experimental protocols required for VMA we first needed to determine a dose-response of the
Calyx of Held to Cd^{2+} ions. Following all other experiments, we needed to exclude the possibility that Ca^{2+} released from intracellular stores could confound our results. To do this we examined the effects of ryanodine (an intracellular Ca^{2+} channel ligand) and thapsigargin (an intracellular Ca^{2+} pump inhibitor) on our experimental protocols. These different experimental protocols are described in detail in the following subsections.

For all experiments we used mice between the ages of 13-20 postnatal days (see the 'The Calyx of Held' section in the previous chapter) and the recordings were made by voltage clamping the cells at – 60 mV.

2.2.1 Cadmium Dose-Response
As described in the Introduction, VMA relies on the manipulation of the probability of release and that this value can be reduced by Cadmium ions (Cd^{2+}). In order however to properly determine the appropriate probability settings the knowledge of a dose-response curve is necessary. To obtain this curve for the Calyx we repeatedly stimulated the cells, 20 seconds apart in progressively increasing Cd^{2+} concentrations (1-50 μM). At each concentration we averaged 10 responses after stabilization of the EPSC amplitude.

2.2.2 Variance-Mean Analysis
In order to make apply the technique of variance mean analysis, the parabolic curve, predicted by the binomial probability distribution, must be estimated. Using the information of the Cd^{2+} dose response curve we followed two different protocols, with 5 points to estimate the parabola in either case.

30
The first protocol included experiments in control ACSF, 1 mM TEA, 5 μM Cd²⁺, 10 μM Cd²⁺ and the origin arbitrarily added. The origin may be included because when the mean amplitude equals zero (i.e. Pr = 0) then by definition the variance of the responses must also be zero. With the addition of TEA this protocol ensured that a large span of the parabola would be covered. In the second protocol the experiments were carried out in control. 3 μM Cd²⁺, 6 μM Cd²⁺, 10 μM Cd²⁺ and again the origin was also added.

In both protocols, once the amplitudes of responses stabilized upon changing the bathing solution, we collected 20 – 70 consecutive stimulations 5 – 20 seconds apart. The means and variances of the peak amplitudes were measured and plotted against each other. A parabola was then fitted to the data points. The value of $c_i^2$ was estimated from the spontaneous miniatures, which we collected from the same cells for three minutes prior to the VMA experiments (for an example see figure 3.5).

2.2.3 Strontium Experiments

With Ca²⁺ completely replaced by Sr²⁺ in the ACSF we collected the responses to 20 – 100 consecutive stimulations. The stimulations were 5 – 20 seconds apart. The amplitude distribution of the desynchronized events was then compared, using the Kolmogorov-Smirnov cumulative histogram test (see for example (Hays and Windkler, 1971)) with that of sEPSCs collected from the same cells (also in Sr²⁺).

2.2.4 High Cadmium evoked-mini Experiments

In order to observe evoked quantal release events the [Cd²⁺] was increased in the bathing solution until we observed 30-40% complete failures (that is the probability of release is so low
that no vesicles are released upon stimulation). The concentration of Cd\textsuperscript{2+} needed was 35 – 50 μM. We collected responses 200 – 700 consecutive stimulations, which were 5 to 10 seconds apart. To eliminate the error in measuring the amplitudes of evoked miniatures, introduced by the stimulation artifacts, we averaged 10 events where complete failures were recorded and this was subtracted from all the traces where a release event was evoked. Then the amplitude distribution of the eEPSCs was compared, using the Kolmogorov-Smirnov cumulative histogram test (see for example (Hays and Windkler, 1971)) with that of sEPSCs collected from the same cells in control ACSF.

2.2.5 Ryanodine and Thapsigargin Experiments

Ryanodine (30 μM) was introduced to the bathing solution for 13 – 15 minutes before experiments were performed. In a different set of cells Thapsigargin (3 μM) was used. The amount of time, allowed for complete wash-in of these intracellular Ca\textsuperscript{2+} channel ligands is consistent with the methods of other investigators (Emptage et al., 2001; Llano et al., 2000) who addressed the question of Ca\textsuperscript{2+} release from intracellular stores.

Upon complete wash-in of the drugs full scale, evoked EPSCs as well as spontaneous miniatures were collected in control ACSF. Then high-cadmium evoked miniatures experiments were performed with the same protocol as in section 2.2.4. The amplitudes of full scale evoked EPSCs were then measured and compared to those that were collected before the introduction of either ryanodine or thapsigargin.
Then the amplitude distribution of the eEPSCs was compared, using the Kolmogorov-Smirnov cumulative histogram test (see for example (Hays and Windkler, 1971)) with that of sEPSCs collected from the same cells (also in ryanodine or thapsigargin).

2.3 Analysis

All the analysis was done on commercially available software. The EPSC recordings were analyzed on Clampfit software (Axon Instruments, Foster City, CA), while for the miniature events we used MiniAnalysis Program (Synaptosoft Inc.). Statistical analyses were done using Microsoft Excel (Microsoft Office 97) and Maple V, using a Pentium II pro computer with 64 MB RAM and the Windows 95 operating system as well as a Pentium II computer with 128 MB RAM and the Windows 98 operating system.

In MiniAnalysis the baseline was averaged for 1.5 ms starting from 2 ms prior to the peak. These values limit the correct detection to only those events which have a rise-time shorter than 0.5 ms. For this reason the events with rise-time longer than 0.5 ms were excluded from all spontaneous recordings of all experiments and quantal eEPSCs recorded in Sr\(^{2+}\) and Cd\(^{2+}\).

The averaged data will be given as mean ± standard error of the mean (S.E.) unless otherwise indicated.
### 2.4 List of Chemicals

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<th>Sources</th>
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<td>Strychnine</td>
<td>SIGMA</td>
</tr>
<tr>
<td>K-gluconate</td>
<td>SIGMA</td>
</tr>
<tr>
<td>CsCl</td>
<td>ACP</td>
</tr>
<tr>
<td>EGTA</td>
<td>SIGMA</td>
</tr>
<tr>
<td>HEPES</td>
<td>SIGMA</td>
</tr>
<tr>
<td>Lidocaine N-ethyl Bromide (QX-314)</td>
<td>TOCRIS</td>
</tr>
<tr>
<td>TEA</td>
<td>SIGMA</td>
</tr>
<tr>
<td>Kynurenic Acid</td>
<td>TOCRIS &amp; SIGMA</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>ALOMONE</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>ALOMONE</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>BDH CHEMICAL</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>SIGMA</td>
</tr>
</tbody>
</table>

Table 2.1 List of Chemicals
3 Results

In this section we present all the data collected in the proposed experiments as well as the results of preliminary work, which was performed to help with the design of the protocols, and some of the justifications.

3.1 Cadmium dose-response

To find the correct Cd$^{2+}$ concentration for the required probability settings we obtained a dose-response curve from 5 cells (Figure 3.2). Sample traces in different concentrations of Cadmium from one of the cells are presented in Figure 3.1.

![Sample traces of EPSCs in increasing extracellular Cd$^{2+}$ concentrations](image)

*Figure 3.1 Sample traces of EPSCs in increasing extracellular Cd$^{2+}$ concentrations*
From the average extent of transmission blockade (Fig 3.3) it's apparent that 50% reduction occurs at $[Cd^{2+}] = 5\mu M$ and at $[Cd^{2+}] = 20\mu M$ there is an approximately 90% reduction in transmission.

**Figure 3.2 Dose-Response to Cd$^{2+}$**

**Figure 3.3 The average dose-response of the 5 cells in Fig. 3.2**
3.2 Variance-Mean Experiments

After we obtained the knowledge of the dose-response curve for Cadmium and before doing the full-scale VMA experiments we first tried to follow a simpler protocol. This method is also based on a binomial probability model but does not require the knowledge of the parabola to allow for the estimation of synaptic parameters. The procedure is as follows. We know that, assuming binomial probability characteristics, the average EPSC amplitude and the variance at any given probability setting is:

\[ x = NpQ \]
\[ s^2 = Np(1 - p)Q^2 \]

Furthermore the coefficient of variation of the amplitude of the EPSCs recorded in consecutive stimulations is:

\[ C.V. = \frac{s^2}{x^2} = \frac{Np(1 - p)Q^2}{N^2 p^2 Q^2} = \frac{1 - p}{Np} \]

Note that C.V. (in equation 1.4 it was denoted as \( c_e \) for the ease of comparison with \( c_b \) which represents the theoretical value of the coefficient of variation for a binomial distribution) the coefficient of variation of the EPSC whereas \( c_n \) represents the coefficient of variation among the quanta. The above equation can be rearranged to:

\[ \frac{s^2}{x^2} = \frac{1 - p}{Np} \Rightarrow \frac{s^2 Np}{x^2} = 1 - p \Rightarrow p = 1 - \frac{s^2 Np}{x^2} \]

but we know that \( x = NpQ \), so

\[ p = 1 - \frac{s^2 Np}{N^2 p^2 Q^2} = 1 - \frac{s^2}{NpQ^2} = 1 - \frac{s^2}{(NpQ)Q} = 1 - \frac{s^2}{xQ} \]
So, in the equation both of s' and x' can be obtained from the consecutive EPSC recordings. If we then estimate the quantal size for the evoked responses with those of the sEPSCs then the release probability of the synapse can be obtained from:

\[ p = 1 - \frac{s'^2}{xQ} \]

These methods have been previously described (Chuhma and Ohmori, 1998). We followed these methods for several cells but obtained negative values for the probability of release which is a mathematical contradiction. For the ‘p’ to be negative, the value of the last term \( \frac{s'^2}{xQ} \) is unacceptably large. This can be due to either the variance of the EPSC amplitudes being too large, the estimated quantal size too small or both.

The variance can be overestimated if the recordings, in addition to the probabilistic variability of EPSC amplitude, erroneously include other sources of the unevenness. Upon closer observation we found that over the time course of the electrophysiological recordings the average of the EPSC amplitude shifted (Fig 3.4). The variance due to this shift was added to the local variations which is the only thing we intended to measure and which represents the different number of quanta recruited on consecutive stimulations. There are techniques to remove this variation by computing local variances (Sigworth, 1980), but instead we decided to include only results of VMA experiments where this baseline drift was not present. A possible reason for this baseline shift is the continual change of the intrinsic series resistance of the recording electrode.
Obtaining negative values for the probability of release also indicated that a possible reason for this error is the underestimation of the quantal size when we use the spontaneous events for this purpose.

**Tendency of Average EPSC Amplitude**

![Graph](image)

**Figure 3.4 An example of the shift of average EPSC amplitude**

On occasion, the EPSC amplitude changes monotonically during the course of the recording. This usually indicates deterioration of the quality of the recording parameters and often is a direct result of the increase in the intrinsic resistance of the recording electrode. The variance of the EPSC amplitudes includes two components. One is due to the tendency of the EPSC amplitudes to have smaller values and the other is the local variations.

After these partial methods we proceeded with the full-scale VMA experiments. The success rate of these experiments was very low (5 out of approximately 30). Due to the lengthy procedure, many cells died before the recordings at all the probability settings could be made. Also a large number of the recordings had to be excluded as a result of the aforementioned baseline drift. Still
for 5 cells we could obtain an estimate of the quantal size. As an example from one of the 5 cells we collected the data in Fig. 3.5.

![Figure 3.5 Results of a sample experiment with recordings used for Variance-Mean Analysis](image)

To verify that the variance observed in each experiment is only due to the variations in the number of vesicles recruited on consecutive stimulations and not to a drifting of the average we made a scatter plot of the amplitude vs. episode number (Figure 3.6)
The variance and the mean for each probability setting were calculated, plotted on a scatter plot and fitted with a parabola (Fig. 3.7).
From this parabola the coefficient of the x-term (for the parabola exemplified this value is 0.0714) represents the slope of the tangent line to the parabola at the origin and this value gives us an indication of the quantal size as described in the introduction.

![Graph showing spontaneous events](image)

**Figure 3.8 An example of a recording spontaneous events**

Spontaneous events occur in the absence of presynaptic stimulation. The average amplitude of these events is generally taken to be a representation of quantal size. Note however the large event which is magnified (inset) to show.

For the estimate of quantal size to be made the value of $c_\alpha^1$ is necessary. We estimate this value for the quanta representing the evoked response from that of the spontaneous events. This
estimate may not be accurate but this is the only estimate available to us. Other investigators have used this method previously. An example of spontaneous miniature recordings is shown in Figure 3.8.

From this figure we see that the average size of all quanta may be around 50 pA. The largest spontaneous event is magnified and presented as an inset. An amplitude frequency histogram of this spontaneous miniature recording is presented in Figure 3.9.

Amplitude Frequency Histogram

![Amplitude Frequency Histogram](image)

**Figure 3.9** Amplitude histogram of the recording in Fig. 3.8

The y-axis represents the number of events. Note that this histogram of spontaneous event amplitudes from Fig. 8 has a single peak.

For this particular cell the coefficient of variation of the sEPSCs was 0.313. Therefore, following the procedure of equation 1.12 we obtain an estimate of the quantal size as
From the figure of spontaneous minis we observe that the average size of miniatures seems to fall below this estimate. In fact the average size for the sEPSCs was 0.049 nA. Both these values belong to Cell 3 in Table 3.1 where results of all the 5 experiments are collected.

\[
Q = \frac{A}{1 + c_m^2} = \frac{0.071}{1 + 0.31^2} = 0.065
\]

<table>
<thead>
<tr>
<th></th>
<th>Q (nA) Estimated</th>
<th>Q (nA) Measured</th>
<th>(c_m)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>0.062</td>
<td>0.051</td>
<td>0.34</td>
<td>555</td>
</tr>
<tr>
<td>Cell 2</td>
<td>0.049</td>
<td>0.041</td>
<td>0.34</td>
<td>322</td>
</tr>
<tr>
<td>Cell 3</td>
<td>0.065</td>
<td>0.049</td>
<td>0.31</td>
<td>286</td>
</tr>
<tr>
<td>Cell 4</td>
<td>0.094</td>
<td>0.079</td>
<td>0.43</td>
<td>196</td>
</tr>
<tr>
<td>Cell 5</td>
<td>0.075</td>
<td>0.059</td>
<td>0.49</td>
<td>123</td>
</tr>
<tr>
<td>Average</td>
<td>0.069 ± 0.0075</td>
<td>0.056 ± 0.0035</td>
<td>0.38 ± 0.033</td>
<td>296 ± 67</td>
</tr>
</tbody>
</table>

Table 3.1 Results of Variance-Mean Analysis Experiments

The values for the coefficient of variation of the sEPSCs fall within the range of values reported by others (Bekkers and Clements, 1999; Chuhma and Ohmori, 1998; Isaacson and Walmsley, 1995). From the table we can also see that the average number of release sites is approximately 300, which is lower than values reported previously.

Treating the estimated and the measured quantal values as two different populations we performed a one-tailed t-test and found the difference in mean amplitudes to be statistically very highly significant (\(p < 0.0001\)) (Fig. 3.10).
While the results of the VMA experiments supported our hypothesis, we further investigated the question with non-model-based methods. We decided to confirm our findings by other means due to the controversy surrounding the statistical treatment applied to transmitter release.

**Spontaneous vs. Variance-Mean Analysis**

![Graph showing comparison between Spontaneous and Variance-Mean Analysis](image)

*Figure 3.10 Average results of the Variance-Mean Analysis Experiments*

**3.3 Strontium Experiments**

The performance and analysis of these experiments are much faster and easier than those in the previous section. Altogether we performed successful experiments in 7 cells (Table 3.2). Figure 3.11 shows the results of the experiment as carried out using one of these cells. In this figure traces of all the stimulations are shown superimposed. We can see the general tendency of the larger quanta occurring right after the stimulation. This tendency can be seen in recordings of single stimulations, as shown in Figure 3.12.
Figure 3.11 Sample traces recorded during the replacement of extracellular Ca\(^{2+}\) with Sr\(^{2+}\).

Figure 3.12 Sample traces from Fig. 3.11 shown individually.

To demonstrate that the tendency of Figure 3.11 is not an aggregate result, here we show individual traces from the same cell.
Figure 3.13 Sample trace showing individual events recorded in Sr²⁺

The early portion (see heavy bar) of the bottom trace from Figure 3.12 is magnified to show individual evoked events recorded in Sr²⁺.

Furthermore, the bottom trace of Figure 3.12 is magnified to show the individual events (Fig 3.13). Comparing these events to that magnified spontaneous miniature event of Figure 3.8 shows the similarity between these eEPSCs and sEPSCs.

In Figure 3.14 an example of spontaneous recordings in Sr²⁺ is also given. From here we can again see that the spontaneous events amplitudes are variable and that the large ones remain.
Figure 3.14 Example of spontaneous events recorded in Sr$^{2+}$

Note that the large, spontaneous events remain when the extracellular Ca$^{2+}$ is replaced by Sr$^{2+}$.

<table>
<thead>
<tr>
<th></th>
<th>Desynchronized Q</th>
<th>Spontaneous Q</th>
<th>Kolmogorov-Smirnov test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>0.075</td>
<td>0.060</td>
<td>p &lt; 0.0000</td>
</tr>
<tr>
<td>Cell 2</td>
<td>0.041</td>
<td>0.038</td>
<td>p = 0.0326</td>
</tr>
<tr>
<td>Cell 3</td>
<td>0.054</td>
<td>0.047</td>
<td>p = 0.0302</td>
</tr>
<tr>
<td>Cell 4</td>
<td>0.045</td>
<td>0.038</td>
<td>p &lt; 0.0000</td>
</tr>
<tr>
<td>Cell 5</td>
<td>0.038</td>
<td>0.030</td>
<td>p = 0.0009</td>
</tr>
<tr>
<td>Cell 6</td>
<td>0.042</td>
<td>0.040</td>
<td>p &lt; 0.0000</td>
</tr>
<tr>
<td>Cell 7</td>
<td>0.043</td>
<td>0.040</td>
<td>p = 0.0075</td>
</tr>
<tr>
<td>Average</td>
<td>0.048 ± 0.0043</td>
<td>0.042 ± 0.0032</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Results of Strontium Experiments

Again, we assume that the spontaneous and desynchronized (i.e. evoked) events represent different population and perform the t-test, which shows a highly significant difference in quantal size (p < 0.01) (Fig 3.15).
At this point we must also mention that the t-test is not the most appropriate measure of significance in this case because we intend to measure the population differences within a cell rather than groups of cells. Hence for each cell we compared the cumulative amplitude distributions using the Kolmogorov-Smirnov test (Table 3.2). As an example see Figure 3.16 for the cumulative amplitude difference for one of the cells. These tests, as expected, also show a statistical significant difference in mean amplitude. We decided to include the results of the t-test for completeness.

It must be noted that the differences between the mean amplitude in this case are smaller than in the case of VMA. This possibly due to the long recordings, required for the desynchronized experiments, which allowed for spontaneous events to contaminate our data. However, in this
case the large number of events in both groups (Desynchronized 521 ± 177; Spontaneous 805 ± 278) allowed for the establishment of statistical significance.

![Graph](image)

**Figure 3.16** Cumulative amplitude distribution of spontaneous (dashed) and evoked (solid) events in Sr²⁺

The y-axis here represents the fraction of the total number of events.

With the results of this experiment, our hypothesis was further supported. However, we felt that the contamination of the miniatures prevented us again from obtaining the most accurate result and performed one more experiment, which we felt was the most accurate and also the most convincing method.

### 3.4 High [Cd²⁺] experiments

Due to the lengthy procedures required, the success rate of the experiments performed in high cadmium concentrations is also low. However, we did collect useful data from 5 cells. An example of these recordings is shown in Figure 3.17.

![Graph](image)

**Figure 3.18** shows examples of individual traces from this experiment. From that figure several
things are apparent. The top trace shows a complete failure, an event where presumably no vesicles are released upon presynaptic stimulation. Another important feature to see is that the largest event of the third trace has a fast rise-time still. The bottom trace shows a double event, which is easily recognizable and these events can be analyzed both, separately, without an overestimation of the amplitude of either one. Finally, although it is hard to see from this figure the second last trace has an inflection point on its rise phase. Due to this inflection its rise-time is too long and this event is excluded from analysis because it’s likely a double event rather then a single quanta.

\[ [\text{Cd}^{2+}] = 40 \, \mu\text{M} \]

Figure 3.17 Sample traces of evoked quantal events in Cd^{2+}
Due to the stimulation artifact the true amplitudes may be hard to measure accurately. We can deal with this problem in two ways. We may calculate the amplitude from the average of the baseline before the stimulation artifact or use the failures to calculate the true baseline during the event. We did both. First we subtracted the average of the failures from all the events. This gave us the true amplitude of the events relative to the baseline of events during the stimulation. Then we used the time prior to the stimulation artifact to calculate the baseline. Figure 3.19 shows the amplitude scatter plot of all the events of this experiment. The failures are easily identified and removed (Fig 3.20).

![Figure 3.18 Individual traces recorded in Cd²⁺](image-url)

Figure 3.18 Individual traces recorded in Cd²⁺
Figure 3.19 Scatter plot of amplitude measurements of evoked quanta in Cd^{2+}

The average of the actual events can then be compared with those of the spontaneous ones (Fig 3.21). The results of all the experiments are shown in Table 3.3.

Figure 3.20 Scatter plot of amplitude measurements after the removal of failure events
Figure 3.21 Scatter plot of amplitude measurements of spontaneous events

The y-axis represents amplitude in nA. Note that the x-axis is labeled with time as opposed to episode number in Figure 3.21. This is due to the fact that for this figure we only plotted the first 50 seconds of a 3 minute recording to make the number of events similar to those in the previous figure.

<table>
<thead>
<tr>
<th></th>
<th>Evoked Q</th>
<th>Spontaneous Q</th>
<th>Kolmogorov-Smirnov test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>0.055</td>
<td>0.037</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Cell 2</td>
<td>0.076</td>
<td>0.046</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Cell 3</td>
<td>0.067</td>
<td>0.043</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Cell 4</td>
<td>0.066</td>
<td>0.047</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Cell 5</td>
<td>0.048</td>
<td>0.039</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Average</td>
<td>0.062 ± 0.0045</td>
<td>0.042 ± 0.0018</td>
<td>p = 0.0001</td>
</tr>
</tbody>
</table>

Table 3.3 Results of experiments in Cd²⁺

As with the results of the Strontium experiments we performed the Kolmogorov-Smirnov test to establish the statistical significance. The cumulative distribution of the sEPSCs and eEPSCs events are shown in Figure 3.22.
Figure 3.22 Cumulative amplitude distribution of spontaneous (dashed) and evoked (solid) events in Cd$^{2+}$

The y-axis here represents the fraction of the total number of events.

However, for the average values of both experiments for all the cells, we again report the t-test results, which were highly significant (0.001 ≤ p ≤ 0.01) (Fig 3.23).

Figure 3.23 Average results of the Cadmium Experiments
At this point we were confident that our observation of the evoked quantal size being larger on the average from those of spontaneous events. However, following the recommendation of Dr. Graham Collingridge, we performed experiments to validate that the difference noted was in fact due to the quantal size and not to a confounding factor of intracellular Ca\(^{2+}\) release. This is important because recently Ca\(^{2+}\) release from intracellular stores has been shown to trigger large quantal events at an inhibitory synapse (Llano et al., 2000) as well as correlate with spontaneous events in hippocampal Synaptic Boutons (Emptage et al., 2001).

### 3.5 Ryanodine and Thapsigargin Experiments

For both the ryanodine and the thapsigargin experiments, first we collected spontaneous miniature- and full-scale EPSC recordings in control solution. Subsequently, we washed in either ryanodine or thapsigargin for approximately 15 minutes.

**Ryanodine wash-in for 15 minutes**

![Scatter plot of EPSC amplitude during wash-in of Ryanodine](image)

*Figure 3.24 Scatter plot of EPSC amplitude during wash-in of Ryanodine*
If intracellular Ca\textsuperscript{2+} is important in evoked release events then using 30 \textmu M ryanodine, which blocks the intracellular Ca\textsuperscript{2+} channels in the closed state (McPherson et al., 1991), should significantly decrease the EPSC amplitude. However this was not observed (Fig. 3.24).

The average of the first and the last 10 episodes were 7.76 and 7.20 nA respectively (Fig. 3.25). After the 16 minutes of ryanodine wash-in we again collected spontaneous miniatures but neither did the average of these events decrease.

![Control vs Ryanodine](image)

**Figure 3.25 Sample traces in Control ACSF and Ryanodine (30 \textmu M)**

After the test for the affect of ryanodine was performed, we proceeded with the protocol for experiments in high Cd\textsuperscript{2+} concentration, as described in the previous section. The cumulative amplitude distribution for this cell is shown in Figure 3.81.
Figure 3.26 Cumulative amplitude distribution of spontaneous (dashed) and evoked (solid line) events in Cd2+ and Ryanodine.

The y-axis here represents the fraction of the total number of events.

Figure 3.27 Scatter plot of EPSC amplitude during the wash-in of Thapsigargin.
Following a similar protocol with 3 μM Thapsigargin, which depletes the intracellular stores by inhibiting Ca\(^{2+}\) ATP-ases of the intracellular stores (Treiman et al., 1998), lead to similar findings (Fig 3.27).

Although in this case there was a slight, transient decrease in the EPSC amplitude the averages of the first and the last 10 episodes were 10.06 and 9.93 nA respectively. Again the miniature size did not decrease. The results of both experiments are presented in Table 3.4, where there is an additional experiment included in Thapsigargin (Cell 2). On this cell the high Cadmium experiment was carried out in the same slice as Cell 1. Consequently the effect of Thapsigargin could not be tested as no experiments in control ACSF could be done.

<table>
<thead>
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<th>Experiment</th>
<th>Spontaneous</th>
<th>Evoked</th>
<th>Kolmogorov-Smirnov</th>
</tr>
</thead>
<tbody>
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<td>Thapsigargin - Cell 1</td>
<td>0.056</td>
<td>0.085</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Thapsigargin - Cell 2</td>
<td>0.047</td>
<td>0.062</td>
<td>p = 0.0001</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>0.059</td>
<td>0.077</td>
<td>p = 0.0000</td>
</tr>
<tr>
<td>Average</td>
<td><strong>0.054 ± 0.0036</strong></td>
<td><strong>0.0077 ± 0.0075</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 Results of the Ryanodine Thapsigargin experiments

Again both the Kolmogorov-Smirnov test as well as the t-test were performed to establish statistical significance (Table 3.4 and Fig 3.28).
From these findings we concluded that at the Calyx of Held, $\text{Ca}^{2+}$ release from intracellular stores is not necessary for either evoked or spontaneous events.
4 – Discussion

We attempted to probe the validity of the Quantal Hypothesis at a synapse of the CNS through the application of several methods, both theoretical as well as experimental. In each case we found that the average size of the quanta used for evoked release is significantly larger than that of those used for spontaneous events. Although each of our methods has its limitations, either in the form of experimental shortcomings or uncertain underlying assumptions, we feel that together these experiments detect this phenomenon reliably because the limitations of one could be overcome or explained by the other methods.

4.1 Variance-Mean Analysis

The experimental protocol for this method of quantal size estimation was designed along the lines of a theoretical model, which assumes binomial probability characteristics for release of neurotransmitter containing vesicles. However, statistical measurements require repeatable experiments, a quality which may or may not hold true at the synaptic level. By ‘repeatable’ one means a synapse where, prior to each of the stimulations, the probability of a given release site is always the same and also that the number of release sites does not change from one stimulation to the next. It is also necessary that at a given stimulation each of the release sites possess a similar set of parameters. That is the sizes as well as the release probabilities are approximately the same for all releasable quanta. Neither the idea of repeatability nor the uniformity among sites has been ascertained. In fact there are many contradictory findings using these methods (Bekkers and Stevens, 1990; Reid and Clements, 1999) and the validity the binomial assumption has been questioned (Barton and Cohen, 1977; McLachlan, 1978; Quastel, 1997; Redman, 1990; Uteshev et al, 2000).
Furthermore during the experiments one observes the postsynaptic response only, which is the confounding result of a large number of variables. At the Calyx of Held – MNTB synapse the exact size and shape of an EPSC is a factor of at least the following variables: \([\text{Ca}^{2+}]_o\), concentration of glutamate in vesicle, size of vesicle, extent of glutamate content release from vesicle on a fusion event, rate of glutamate removal from the synaptic cleft, type of postsynaptic receptors, number of postsynaptic receptors at a given site. The presence of such a large number of factors would require a multivariate statistical analysis, if at all possible.

Despite all the limitations described above, VMA has been applied to a large number of central synapses and the form we used (Clements and Silver, 2000) was the most recently developed refinement. In simulations this method reliably detected pre or postsynaptic modulation. With the help of the preliminary experiments, (presented in detail in "Results"), we refined our experimental protocols and we are confident that if the method is applicable at this synapse then our results are reliable.

Once we decided on the inclusion of the results of these experiments we mention a few advantages and limitations. Most importantly the theory predicts deviations from the parabolic curve in case of variability in \(P_r\) among the release sites, which allows for an intrinsic check on the validity of binomial settings at this synapse. In particular the deviations would be apparent at high values of \(P_r\) (i.e. at the rightmost, falling phase of the parabola). With the inclusion of experiments with TEA in our protocols we were able to obtain data points in this region of the parabola. The parabolic fits were excellent and this suggests uniformity in \(P_r\) among the sites.
There are however 2 limitations of this method (even if the theoretical model is accurate), both of which are similar in origin but intrinsic to the method. One limitation is that we obtain only a single value for the estimated quantal size in a given cell. We must compare this value to that measured from hundreds of spontaneous events from the same cell. This however is statistically meaningless. Therefore the only possible method for establishing statistical significance is among the cells using the t-test. This is not entirely accurate as it measures the population difference among cells rather than within cells. The other weakness results from the fact that the spontaneous events are analyzed with a different method from those of the evoked ones. While the quantal size of evoked events is calculated using the VMA the spontaneous events are actually detected and averaged. This carries in itself the drawback of possible event-detection error in one but not the other. For example, suppose the Minianalysis program, which we used for the detection of spontaneous events, underestimates the amplitude by 5% on the average. This error could artificially create the difference we hypothesized to find. We tried to eliminate this possible source of error by individually and manually detecting every spontaneous event that we included in the analysis.

All in all, VMA is our least reliable method. It is presented here first to keep the timeline of experiments in order. We consider these results an indication of possible mean size difference between sEPSCs and eEPSCs quanta.

4.2 Strontium Experiments

Using Strontium ions to estimate the quantal size of evoked events by desynchronizing the quanta included in an EPSC has been done previously, in dentate granule neurons (Bekkers and Clements, 1999), at the neuromuscular junction (Raman and Trussell, 1995), in cultured
hippocampal neurons (Abdul-Ghani et al. 1996) and the chick auditory brainstem instead (Otis and Trussell, 1996).

One limitation of this experiment is the length of recordings required. While the average EPSC of an MNTB neuron lasts only a few milliseconds, desynchronized quantal events can be observed for a much longer time period. For this reason the recordings lasted approximately 800 ms with the stimulation delayed 100 ms. This length of time however is long enough for several miniature events to occur. Thus the evoked events were contaminated by spontaneous ones. See for example the traces in Fig. 4.1 where sEPSCs are recorded even during the first 100 ms of the recording, before the stimulation.

![Figure 4.1 Sample traces in Sr^{2+} showing the contamination of measurements by spontaneous events](image)

Note the large number of events during the first 100 ms (i.e. before the stimulation artifact). The high rate of spontaneous events continues after stimulation, which contaminates our results as we intend to measure the amplitude of evoked quanta only. Note however that the events before stimulation tend to be smaller than those afterwards.
As a result, when we calculated the average amplitude of evoked quanta, the average was
deflected toward those of the inevitably included sEPSCs. However, for this experiment both the
sEPSCs and eEPSCs could be detected and calculated by the same methods. Thus, while error in
the detection method of sEPSCs could artificially produce the quantal size difference in the
VMA method, the results of the strontium experiments are free of these problems.

4.3 High Cadmium Experiments

Cadmium ions have been used previously to observe quantal events. For example Isaacson and
Walmsley used a Cd\(^{2+}\) concentration of 100 \(\mu\)M (Isaacson and Walmsley, 1995). From our
Cadmium dose-response information, we found that a concentration of between 35 – 50 \(\mu\)M
produces about 30 – 40\% complete failures, which we found appropriate for observing quantal
events.

We assume here that the events collected and included for analysis are mono-quantal events.
That is every event is due to the release of a single vesicle. For the experiments carried out in the
presence of high [Cd\(^{2+}\)] we are able to prove this argument analytically. To justify our
interpretation that unitary release events in Cd\(^{2+}\) represents monoquantal release, we estimated
the release probability at an individual release site based on the following derivation.

Let \(S\) = number of consecutive stimuli

\(f\) = number of complete failures

\(N\) = number of vesicle release sites at a given synapse

\(P\) = probability of release at a given site

\(x\) = number of vesicles released at a given stimulation

Knowing the failure rate and assuming a binomial probability model
we can calculate the probability of release at an individual release site.

Keeping the failure rate at 30-40% by the application of Cd\textsuperscript{2+} allows for a very low probability but still a large number of evoked events. For example in one of our experiments we observed 170 complete failures upon 500 consecutive stimuli (\(N = 500, f = 170\)). For the failures \(x = 0\). Therefore,

\[
P(X = 0) = \frac{f}{S} = \frac{170}{500} = 0.34 = \binom{N}{0} P^0 (1 - P)^{N-0} = N(1 - P)^x
\]

Hence we need to solve 0.34 = \(N(1 - P)^x\) for \(P\). From variance mean analysis we found that on the average the minimum number of release sites was approximately 300 (Table 1). Using this value for \(N\) and solving the equation gives \(P = 0.0036\) for the release probability of a given site. It should be noted that the VMA only indicates a minimum number of release sites. As a result, this value for the probability of release is likely an overestimation.

One criticism may be that even with this low value for \(P\) there is still a finite probability of two or more events occurring at the same time. In fact there is a 29% chance that this would happen. However we are confident that this 29% is not included erroneously. First of all, in this 29% are included events with 3, 4, 5...300 events but we never see events with amplitudes exceeding 4 or more quantal sizes. Secondly, sometimes we see events that are identifiably made up of two closely released quanta (see for example the bottom trace in Fig. 3.18) but we have not observed 3 or more quanta released in this manner. Finally, many events can be identified to be multiquantal because two the rise-time or decay-time is/are slower than average (which would
indicate poor synchronization of more than one quanta). All the events with multiple peaks or long rise/decay times are excluded. Hence the 29% fraction of possible multiquantal events is not the error level of our experiments. In fact we excluded 51 (10%) events due to too long rise-times and 33 (6.5%) double events. There were 201 single quantal events included. The events not accounted for so far were excluded because of rise times much longer than 0.5 ms. We considered these cases as bad detection rather than multiquantal to avoid bias. However they may have been multiquantal and that would further reduce the margin of error.

We require the rise-time of all events (regardless of amplitude) to be no longer than 0.5 ms (for details see Methods). For example, we plotted the rise-time vs. amplitude of all the evoked events included for analysis for one of the high [Cd²⁺] experiments (Fig. 4.2). Should the larger events contain more than one quanta, we should see a definite positive correlation because then it is expected that larger events should have a longer rise time (Wall and Usowicz, 1998).

However, this correlation is not present. In fact the rise-times of the largest events fall in the average of the all events. A similar plot for the spontaneous events supports the same conclusion (Fig 4.3).

![Figure 4.2 Scatter Plot of rise-time vs. amplitude of evoked events in Cd²⁺](image-url)
The limit set on the rise-time of events was not arbitrary. It was chosen because using the Minianalysis program for events occurring at the Calyx of Held – MNTB synapse this value proved to be the most efficient for proper detection. However, this value is by no means a source for error. Other investigators use a rise-time cut-off of about 0.4 ms for events that have a mean quantal size of $12.8 \pm 0.4 \text{ pA}$ (Wall and Usowicz, 1998).

Another possible source of error may due to our preparation and the limitations of electrophysiological experimentation. Postsynaptic signals are thought to travel passively from the site of the synapse to the axon hillock, where the actively spreading action potential is generated in the postsynaptic cell. As passive electrical signals travel they tend to be distorted. One major signal degradation problem is cable filtering, where the EPSCs generated at dendrites distort by the time they reach the soma, where electrophysiological detection may take place (Johnston and Wu, 1995). Although cells of the MNTB are devoid of dendritic inputs, due to the sheer size of the cell body there is a variable distance from the individual synaptic sites to the place where the tip of the electrode actually punctures the cell. Events traveling from farther...
away may be attenuated and hence lead to an underestimation of mean quantal size. It is especially important if our hypothesis is correct and mean and evoked events represent two different sets of release sites. As signals degrade, they not only attenuate in amplitude but also tend to obtain a longer decay time.

![Figure 4.4 Scatter Plot of decay time vs. amplitude of evoked events in Cd²⁺](image)

**Figure 4.4 Scatter Plot of decay time vs. amplitude of evoked events in Cd²⁺**

Hence, on a plot of decay-time vs. amplitude we would expect to find a correlation with smaller events tending to have longer decay times. We made these plots for both the sEPSCs as well as the eEPSCs (Figs. 4.4 and 4.5).
Figure 4.5 Scatter Plot of decay time vs. amplitude of spontaneous events in Cd^{2+}

From both these plots it is apparent that no such correlation is present. And hence the recordings at the Calyx of Held – MNTB synapse reliably represent the true amplitude of both sEPSCs and eEPSCs.

At this point we must mention one limitation that arises from the way we analyze quantal events. In the methods section we described that quantal events are analyzed using the MiniAnalysis program and that we only included events with rise-times of 0.5 ms or less (Fig. 4.2 and 4.3). This exclusion of a large number of events may prevent us from detecting a correlation between the amplitude and the rise-time. This fact may be a criticism of the analysis. We may counter these criticisms in two different ways. First by stating that at least for the events included, the larger events did not tend to have longer rise times even though we included events with the full scale of amplitudes. If there is a correlation then excluding events with longer rise-times should also exclude the events with larger amplitudes. Secondly, we would expect that events with longer rise-times would also possess longer decay-times. That is, rise-times and decay-times are correlated. But neither we do not see a correlation in the amplitude vs. decay-times plots even though those plots include a larger range of decay-time values. Hence we may speculate with confidence that the amplitude vs. rise-time correlation would not be present even if our methods of analysis was different and events could be included with longer rise-times.

As we mentioned previously these experiments provide our most compelling evidence and probably the most accurate measurement of the true quantal size of evoked events.
4.4 Further Justifications

As a further verification of the results of the two experiments where the same methods for detection and measuring could be used for both the sEPSCs and the eEPSCs we intended to compare the amplitude frequency histograms of evoked and spontaneous events by other means than the Kolmogorov – Smirnov test. One way to do this is to calculate the percentage of events that fall above the average. Table 4.1 summarizes the results of these calculations.

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous</th>
<th>Evoked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strontium Experiments</td>
<td>48.69 ± 2.77</td>
<td>76.20 ± 3.05</td>
</tr>
<tr>
<td>High Cadmium Experiments</td>
<td>40.06 ± 3.79</td>
<td>50.96 ± 4.34</td>
</tr>
</tbody>
</table>

Table 4.1 Percentage of events larger than the average amplitude

From these percentages it is clear that the averages of evoked responses were higher because of the recruitment of more large quanta.

Moreover, if all of large events recorded, were chance occurrences of quanta, coincident in time then we expect to see several peaks in the amplitude histogram plot (Wall and Usowicz, 1998). The peaks should presumably occur evenly spaced with the distance between the peaks representing the quantal size. This, however, we never observed. Our amplitude histograms of miniatures (see for example Fig 3.9) were unimodal for spontaneous and evoked events. Hence we are confident that our events represent single quanta.
4.5 Further Discussion of the Model of Synaptic Transmission

As we pointed out in the introduction, the Quantal Hypothesis was derived based on experiments at the neuromuscular junction. It is however not clear if the ideas of the Quantal Hypothesis apply directly and unconditionally to synapses in the CNS. In fact there is a large body of evidence, using different methods, indicating that, in the CNS, spontaneous events are different from those of evoked ones. In this thesis we sought to address the possibility that the eEPSCs are larger than the sEPSCs. The results of our experiments and analyses further support this body of evidence and help build a more complete model of synaptic transmission.

In the section outlining our hypothesis we listed several lines of work, which hinted on the need for expanding the model of synaptic transmission. Now, we again follow this list and in the light of our results try to propose a slight but significant expansion to this model.

Bekkers and Clements have observed previously that the average amplitude of eEPSCs was larger than that of sEPSCs at the rat dentate granule neurons. Our results indicate that their findings are not an anomaly in the CNS.

The so-called “kiss-and-run” release mechanism has received a lot of attention lately. Based on our work one may propose that evoked and spontaneous events perhaps use different vesicle fusion processes.
The work demonstrating the existence of different docking complexes for the sEPSCs and the eEPSCs seems to strengthen the possibility of, not only a different fusion mechanism for these processes, but also two different pools of synaptic vesicles, each existent perhaps for different purpose in the physiology of synaptic transmission.

The idea of the spontaneous and evoked events performing different roles at a synapse is strengthened by the finding, that in the absence of presynaptic stimuli, the neurotransmitter receptors are removed from the postsynaptic cell membrane. From this evidence one may speculate the likelihood of sEPSCs serving a role of synaptic maintenance as opposed to eEPSCs, which are mainly responsible for the transmission of signals.

The separation of synaptic maintenance mechanisms from those of transmission seems a reasonable proposal, based on the results of this study, which offers a physiologically sensible model of synaptic transmission, for many parts of the nervous system. Using a separate set of quantal units for triggered transmission may be especially important in neurons of the auditory pathway which is specialized for the transmission of high frequency stimuli. The reason is that due to the larger average amplitude of evoked events, a full scale EPSC can be obtained with fewer quanta recruited.

4.6 Conclusion

Based on the results of the three different lines of experiments we conclude that at the Calyx of Held – MNTB synapse the evoked events contain quanta which have a significantly larger average amplitude than spontaneous events. This conclusion seems to be valid regardless of whether a simple binomial probability model adequately describes the release events of synaptic
vesicles. The methods of Variance-Mean Analysis, Strontium ion induced disynchronization and unitary EPSCs evoked in the presence of Cadmium ion showed the evoked quantal size to be larger by 24%, 16% and 47% respectively. Our results do not contradict the ideas of the Quantal Hypothesis but they do introduce heterogeneity into the physiology of the nervous system. Our proposal of synaptic transmission offers a physiologically sensible model for many parts of the nervous system, but it may be especially important at the auditory pathway, which is specialized for the transmission of high frequency stimuli.

4.7 Possible Future Directions

There are several different lines of work that one may pursue based on the work presented here. These include different methods of analysis of the collected data, confirming the results of these experiments using different methods and/or different preparations as well as testing the validity of the model suggested.

First of all the collected data is available for further analysis and representation. One could obtain valuable information from the analysis of changes in the EPSC amplitude and variance. Also, recent advancements in the mathematical description (Mendelbrot, 1982) of fractals and the realizations that many physiological systems contain fractal characteristics (Bassingthwaighte J.B. et al., 1994) may provide a direction, which is worth the effort. For example the spontaneous miniature recordings may contain characteristics, which could be elucidated by the application of fractal and/or Fourier transformation techniques.

There are several experiments, which would verify our conclusions. These would be an important foundation for other investigations regarding the physiology of the Calyx of Held –
MNTB synapse, general synaptic transmission and learning and information processing in the nervous system. Other investigators may have different interest ranging from the specific physiology and pathology of the auditory pathway to clinical studies of auditory disorders and treatments for them.

In our opinion the most suitable experiment would be to progressively increase the \([\text{Cd}^{2+}]\) from that which produces 30 – 40% failures to as high levels as are experimentally possible. Should the reason for larger events in our recordings be the coincident release of multiple quanta, a reduction of \(P_r\) (i.e. an increase in \([\text{Cd}^{2+}]\)) should reduce the frequency of larger events and hence the average amplitude. Due to the large number of failures in high \(\text{Cd}^{2+}\) concentrations, it may not be feasible to collect a large enough number of quantal events to allow for statistical comparison. In that case one may follow the procedure followed by Isaacson and Walmsley when they used a pair-pulse stimulation on the endbulbs of Held in the anteroventral cochlear nucleus of the rat (Isaacson and Walmsley, 1995). In that case almost every pair-pulse stimulus results in an event. However, this method would mix up basal release probabilities with those obtained after pair-pulsed facilitation and this may be a criticism of the method.\(^70\)

Finally as we mentioned in our introduction the general progression of science occurs in the stepwise conceptualization of models. The model we suggested seems sensible for the findings of this study and for the results reported by several other scientists. The validity of this model is hard to predict at this time. It is however surely not a complete representation of synaptic transmission and possibility future work based on our proposal is immense.
Bibliography


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