EVIDENCE FOR PRESYNAPTIC PROTEIN AT THE CALYX OF HELD NERVE TERMINAL

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

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New Brunswick, New Jersey

January, 2018
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Presynaptic activity requires the localization and maintenance of thousands of proteins that are typically thought to be synthesized in the soma and transported to nerve terminals. However, local protein synthesis occurs at dendritic locations and local presynaptic protein synthesis has recently been shown to occur in an inhibitory neuron in the brain, but the small size of most nerve terminals complicates further studies. Here, we study presynaptic protein synthesis at the calyx of Held nerve terminal, located in the mammalian brain. The size of this terminal, its long axon, and high firing frequency make it an ideal choice to study presynaptic protein synthesis. We show a major ribosomal component, 5.8S ribosomal RNA is present in this presynaptic nerve terminal. To verify the presence of functional presynaptic ribosomes, we used the surface sensing of translation (SUnSET) technique. This produced well-defined fluorescent signals in presynaptic
terminals and postsynaptic cell bodies, and the fluorescent signal was eliminated by inhibiting protein synthesis. To determine the effects on synaptic transmission, we measured electrical activity. After inhibiting translation, the initial frequency of spontaneous events increased by ~2-fold but the amplitude was unaffected, indicating a presynaptic mechanism. In addition, we find that evoked responses show less depression during high frequency firing (≥ 100 Hz). The reduction in depression is not consistent with effects on desensitization but is well explained by presynaptic changes in neurotransmitter release. These findings further indicate that presynaptic protein synthesis occurs, that it can affect spontaneous and evoked release of neurotransmitter, and it affects neurotransmitter release at high firing frequencies.
Dedication

For my grandmother

This dissertation is dedicated to my family but specifically my grandmother Fay Hotchkiss. My grandmother was the most loving, caring and smartest person I was ever fortunate to have a relationship with. She instilled in me at a very young age that hard work and treating people with respect will carry you a long way in life. Her compassion and selflessness was contagious, and everyone that met her loved her. I do not think it would have been possible for me to be where I am today if it wasn’t for her love and support. I will never forget you and not a day goes by that I do not think about you. If there were more people like my grandmother in the world, there is no doubt in my mind it would be a much better place. Until we meet again...

To my mother, father, brother, sister, sister-in-law and nephews you are the reason that any of this even matters. I can say with 100% certainty if I didn’t have family like you I would not be where I am today. Your constant love and support made all of this possible. I could never repay you for all that you have done for me. I love each and every one of you.

To my girlfriend Ashley you are the most amazing person I have ever met. You always make sure that I stay grounded and are a constant reminder of the things that really matter in life. You are a special person and I love you very much.
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CHAPTER 1

Introduction

A fundamental process of the auditory system is the localization of sound. Precise encoding of temporal information is critical for animals to localize where a particular sound originates. The calyx of Held is an exceptionally large axosomatic relay synapse that resides in the auditory brainstem. The main requirement to be classified as a relay synapse is the ability to faithfully release neurotransmitter even at elevated presynaptic firing frequencies. The giant size of the calyx of Held allows the harboring of many individual release sites, ultimately permitting the calyx to drive its postsynaptic counterpart, principal cells located in the medial nucleus of the trapezoid body (MNTB), at elevated frequencies. Principal neurons in the MNTB are inhibitory, whose main function is to deliver well-timed and constant inhibition to other brainstem nuclei. The calyx of Held is extremely popular among synaptic and cell physiologists due to ability to easily access the calyx and/or its postsynaptic partner, the MNTB, with a patch electrode. The ease of patch clamp recordings, combined with the ability to unambiguously identify the calyx of Held, especially at early stages of development during the second week after birth, has resulted in this synapse becoming well characterized. In the following sections I will discuss how the calyx of Held functions in sound localization, and the developmental changes that construct the calyx into an ideal relay synapse.
CHAPTER 1.1

Main Function of Calyx of Held Synapse

The calyx of Held is the main synaptic input to principal neurons located in the MNTB. To appreciate how the calyx is a master at sound processing we must first understand the principal neurons. Anatomically, the principal neurons of the MNTB are located ventromedial to the medial superior olive (MSO), positioned within the axons of the trapezoid body (Figure 1 1). Interestingly, each principal neuron is contacted by a single presynaptic nerve terminal, the calyx of Held, resulting in the firing properties of the MNTB being almost completely controlled by calyceal inputs. This single enormous, axosomatic (1:1) input evokes large, glutamatergic excitatory postsynaptic potentials (EPSPs) 4,5. The calyx of Held synapse originates from globular bushy cells (GBCs) of the anteroventral cochlear nucleus (aVCN) (Figure 1 1) 6. The large caliber calyceal axon that projects from GBCs, travel a rather long distance (few mm) and ultimately synapse onto neurons in the contralateral MNTB.

The majority of neurons in the MNTB are excited exclusively by contralateral sounds 7. This is mainly due to the dominant calyceal input that principal neurons receive. The large caliber calyceal axons have a minimum sound response latency to principal neurons of ~4 msec, mainly due to the heavily myelinated GBC axon 8-10. Principal neurons in the MNTB are tonotopically organized, where representation of lower and higher frequency sounds are positioned lateral and medial, respectively.
The calyx of Held synapse fails on rare occasions, so it can be assumed that response of stimuli in the principal neurons mimics that of the GBCs.
CHAPTER 1.2

Main Projections of Principal Neurons

The principal neurons of the MNTB are classified as an inhibitory cell type, more specifically glycinegic. This allows principal neurons of the MNTB to inhibit several ipsilateral nuclei both inside and outside the superior olivary complex (SOC) 6. To describe the main function of the MNTB, I am going to focus on its most prominent target, the lateral superior olive (LSO). Neurons located in the LSO are powerfully inhibited by sound stimuli arriving from the contralateral ear via principal neurons in the MNTB. In addition to the inhibitory input contacting the LSO, there is an excitatory input that arrives from the ipsilateral ear, directly from the cochlear nucleus. Due to the large size of the GBC axon and the minimal synaptic delay at the calyx of Held, inhibition from the contralateral ear lands at approximately the same time as excitation from the ipsilateral side 11. Neurons located in the LSO will then compute intensity differences between both ears determining the localization of sound.

The chief function of the MNTB is to provide well-timed inhibition to many other brainstem nuclei, mainly the LSO. The precise timing and ability for LSO neurons to detect miniscule differences in interaural arrival times would not be feasible if it weren’t for the calyx of Held. There are numerous specializations that allow the calyx of Held to be both reliable and fast throughout its development. Furthermore, the design and structure of the calyx implies that speed and fidelity were the main functions when this synapse evolved. The axosomatic location of this
synapse on to MNTB neurons reduces the time require to generate and trigger and action potential (AP) in distal axonal areas.
CHAPTER 1.3

Dynamic Development of the Calyx of Held Synapse

The calyx of Held has a sizeable number of synaptic vesicles that can be liberated, in response to a presynaptic AP. This allows a single input the ability to trigger a spike, reducing fluctuations in arrival time compared with a postsynaptic neuron that is contacted by multiple inputs. The principal neurons in the MNTB rely on calyceal contact for their proper development and survival\(^{12,13}\). The earliest detectable synaptic contacts between the calyx of Held and MNTB are \(\sim\) embryonic day 17 (E17) (Figure 2, left)\(^ {14}\). Progressing through development at \(\sim\) postnatal days 2-3 (P2-3) a preexistent somatic contact further expands into a structure known as the protocalyx (Figure 2, middle). As complex as the adult morphology of the calyx of Held is, the complete formation of the calyx synapse resembles the process most synapses undergo to fully mature, as seen in other areas of the brain. However, with calyx formation the mature form is highly unique due to the full area of innervation being completely restricted to the cellular soma of the principal neuron (Figure 2, right).

The calyx of Held nerve terminal and its postsynaptic partner are both accessible with a patch pipette (Figure 3). This allows for a detailed understanding of the input-output relationship through development, and following complete maturation. Until briefly before hearing onset (\(\sim\)P10) the physiology of the calyx exhibits slow alterations that occur over several days, including a decrease in the duration of the presynaptic AP, the postsynaptic currents and the synaptic delay\(^ {15-}\)
However, more extreme changes occur just before hearing onset. The next sections are going to dissect the major physiological and morphological characteristics that are influenced prior to hearing onset.

The presynaptic action potential generated at the calyx of Held is brief, and trailed by a long lasting depolarizing after potential. To overcome the large capacitive load of this giant presynaptic terminal, the axonal heminode is rather long and peppered with a high density of Na⁺ channels. The terminal itself does not harbor any Na⁺ channels, which contributes to an AP with a shorter half width. However, low and high threshold K⁺ channels are located presynaptically. Transformations in both K⁺ and Na⁺ channels result in a developmental increase in the speed of the presynaptic AP. For example, mature presynaptic Na⁺ channels recover quickly from inactivation. Presynaptic K⁺ channels between (~P7 and 14) become denser with low threshold Kv1 channels positioned at the axonal heminode. In addition to Kv1 channels, Kv3 channel density also increases. These channels gate rapidly but inactivate slowly, and are absent from the release face. The increase in Kv3.1 channels, specifically, contributes to the decrease in the width of the presynaptic AP.

The presynaptic action potential dictates how much calcium influx is going to occur in a nerve terminal. The upward swing of the AP will start to activate presynaptic Ca²⁺ channels, with little influx mainly due to the opposing driving force for Ca²⁺ to enter the neuron. However, along the downward swing of the AP, influx of Ca²⁺ is maximal due to more channels in the open state, and optimal driving force
for the divalent cation. Early in calyx development, the presynaptic AP opens a large portion of Ca\(^{2+}\) channels during the repolarization phase. The immature presynaptic AP is much broader compared to later in development resulting in a longer repolarization phase and more Ca\(^{2+}\) influx. Calyx of Held development, up to the onset of hearing, shows a progressive increase in the size of the presynaptic Ca\(^{2+}\) current. This is a result of the calcium channel composition changing throughout development.

The immature (<P10) calyx of Held is a combination of N-, R-, and P/Q-type Ca\(^{2+}\) channels. The coupling of P/Q-type Ca\(^{2+}\) channels and transmitter release is extremely efficient, due to their positioning inside active zones. Around hearing onset neurotransmission becomes exclusively dependent on P/Q-type Ca\(^{2+}\) channels.

The calyx of Held has a large number of active zones that are corralled into one, compact and well-defined structure. As unique the morphology of this terminal may be, neurotransmitter release properties are similar to other traditional nerve terminals. The calyx of Held has ~300-700 active zones and can release up to 100 synaptic vesicles with a single presynaptic action potential. As stated above, neurotransmitter release is dependent on the amount of calcium that enters the terminal, and this relationship is non-linear. Finally, as with other excitatory synapses the Ca\(^{2+}\) sensor that mediates vesicle fusion is synaptotagmin-2. We have discussed what makes the calyx of Held synapse fast, but what makes it reliable?
CHAPTER 1.4

**Precision and Fidelity of the Calyx of Held Synapse**

During periods of high synaptic activity the calyx of Held has to be fast and reliable to function as a sensory relay synapse. During times of high spiking rates the synaptic delay of the calyx can increase substantially \(^{32-36}\). Nevertheless, the mature calyx of Held synapse exhibits few transmission failures. However, synaptic failures have been observed at this synapse under extremely high firing frequencies, stimulated electrically \(^ {34}\). Independent of high firing frequencies this synapse rarely fails. Prior to hearing onset failures are still rare, but more likely. This is mainly a consequence of phase locking and short-term synaptic depression.

Mechanisms accounting for short-term depression at the calyx of Held have been extensively studied using brain slice electrophysiology. There are three main mechanisms that have been attributed to causing short-term synaptic depression. These are exhaustion of the pool of synaptic vesicles with a high probability of release \(^{37,38}\), inactivation of presynaptic Ca\(^{2+}\) channels \(^{39,40}\) and principal cell receptor desensitization \(^{28,41,42}\). The contribution of each of these mechanisms is highly reliant on stimulus patterns and the developmental stage. Short-term synaptic depression at this synapse is offset by short-term facilitation events, in brief intervals (msecs).

Short-term synaptic facilitation can be observed in almost every neuron prior to hearing onset \(^ {15}\). Facilitation, or synapse strengthening, is described as an increase in the magnitude of a response that lasts for a matter of tens to hundreds of
milliseconds. Mechanistically, residual Ca\textsuperscript{2+} from a prior release event increases synaptic vesicle release probability \textsuperscript{43}. Release probability can be amplified due to increased Ca\textsuperscript{2+} influx caused by faster activation of P/Q-type Ca\textsuperscript{2+} channels \textsuperscript{44-47}. Moreover, residual Ca\textsuperscript{2+} can combine with Ca\textsuperscript{2+} entering the terminal, during subsequent action potentials, leading to an increase in the local Ca\textsuperscript{2+} concentration in proximity to synaptotagmin-2 \textsuperscript{48}. Another possible mechanism to account for short-term synaptic facilitation are endogenous calcium buffers becoming bound by residual Ca\textsuperscript{2+} rendering them inactive for the next influx event \textsuperscript{49}. Taken together the main mechanism(s) accounting for short-term facilitation relies on residual presynaptic Ca\textsuperscript{2+}.

The numbers of vesicles that can be released during an AP depend on the readily releasable pool (RRP), and this is true for any synapse. The RRP size at the calyx of Held synapse has been estimated to be somewhere between 700 and 5,000 vesicles \textsuperscript{37,41,50-53}. Most vesicles docked at the calyx of Held active zone are readily releasable. Recovery from short-term depression can be represented by replenishment of the RRP and an increase in the probability of release. The calyx of Held contains synaptic vesicles that have the ability to rapidly replenish thus sustaining synaptic transmission at high firing frequencies.
CHAPTER 1.5

**Developmental Progression is Accompanied by Changes in Short Term Synaptic Plasticity**

Firing frequency and pattern gradually change throughout calyx development, with the most drastic morphological and physiological changes occurring just prior to hearing onset \(^{15}\). On average, the firing frequency is much higher following hearing onset, with little change in the properties of short-term plasticity \(^{15,16}\). The three mechanisms impacting short-term depression, as discussed above, are greatly reduced following hearing onset. The most significant developmental change is a reduction in the probability of release, which is mainly caused by a sharpening in the presynaptic AP \(^{17}\). A faster action potential results in Ca\(^{2+}\) transients that are short-lived and smaller in magnitude \(^{54}\). Furthermore, a reduction in the affinity of the Ca\(^{2+}\) sensor can also contribute to a decrease in the probability of release \(^{54,55}\). Following the onset of hearing, the quantity of active zones increase, with a reciprocal decrease in their size. The number of docked vesicles, however, remains unchanged resulting in a net decrease in the number of docked vesicles per active zone (see Figure 2) \(^{28}\). There are many compensatory mechanisms that combat the decrease in the number of vesicles available for release, for example an increase in the RRP \(^{17,28,56}\).

Calcium concentration in the presynaptic nerve terminal is described using “domains”. Activation and opening of presynaptic Ca\(^{2+}\) channels creates a gradient of increased intraterminal Ca\(^{2+}\) concentration, which is termed a Ca\(^{2+}\) nanodomain.
An example of the domain models for intracellular Ca\(^{2+}\) concentrations can be illustrated in a situation where Ca\(^{2+}\) channels are located in close proximity to one another. In this example the channels Ca\(^{2+}\) nanodomains will now overlap creating a microdomain of elevated Ca\(^{2+}\). Several studies suggest that most immature calyces posses synaptic vesicles that are oriented towards a Ca\(^{2+}\) microdomain \(^{18,24,30,48,57}\). This would indicate a loose coupling of presynaptic Ca\(^{2+}\) channels and synaptic vesicles early in calyx development. Presynaptic scaffolds are mainly responsible for the coupling of synaptic vesicles with Ca\(^{2+}\) channels, for example (regulating synaptic membrane exocytosis protein) RIM \(^{58}\).

Following the onset of hearing, the distance between Ca\(^{2+}\) channels and synaptic vesicles tighten \(^{28}\). This is due to a change in the Ca\(^{2+}\) channel subtype and their propensity for coupling. Immature calyces contain a mixture of Ca\(^{2+}\) channel subtypes, composed of N-, R- and P/Q type channels. After hearing onset, the more loosely coupled Ca\(^{2+}\) channel subtypes (N and R) become replaced with higher coupling type Ca\(^{2+}\) channels, P/Q \(^{25}\). The P/Q-type Ca\(^{2+}\) channels couple more efficiently to neurotransmitter release resulting in an increase of exocytic proficiency. The tighter coupling seen following hearing onset offsets the reduction of presynaptic Ca\(^{2+}\) influx caused by a shorter AP half width \(^{54,55}\).

A second type of developmental change to combat the effects of short-term synaptic plasticity is an elevation of intraterminal calcium buffers. This results in an increase in Ca\(^{2+}\) clearance \(^{59}\), and a decrease in inactivation of presynaptic Ca\(^{2+}\) channels \(^{28,60}\).
The third mechanism that contributes to short-term depression, postsynaptic receptor desensitization, is greatly reduced following hearing onset. The lower probability of release seen after hearing onset ultimately results in a decrease of glutamate accumulating in the synaptic cleft and faster glutamate clearance, allowing AMPA receptors to recover more quickly from desensitization \(^{28,61-63}\). The drastic change in calyx morphology (increased surface area) throughout development can account for more efficient glutamate clearance (see Figure 2) \(^{64,65}\).

A reduction in release probability accounts for a decrease in synaptic depression, throughout maturation of this synapse. After the onset of hearing there is also a decrease in synaptic facilitation. The main cause of this is a decrease in the intracellular residual \(\text{Ca}^{2+}\) concentration due to more efficient clearing \(^{59}\). Increased competence of \(\text{Ca}^{2+}\) clearing is directly correlated with an increase expression level of calcium binding proteins throughout calyx maturation \(^{64,65}\). Taken together, the calyx of Held exhibits numerous morphological and physiological changes throughout development that fully ensure maturation into a reliable sensory relay synapse.
CHAPTER 1.6

Calyx of Held Summary

Sound localization is a fundamental process mediated by many nuclei located in the auditory brainstem. The main function of principal neurons of the MNTB is to provide well-timed inhibition to several other brainstem nuclei. The calyx of Held synapses with individual principal neurons located in the MNTB with a single, large, axosomatic contact resulting in a 1:1 input-output relationship. The calyx of Held can drive a principal neuron with fidelity and precision before hearing onset, and even more so following the onset of hearing. This nerve terminal is desirable to many synaptic physiologists due to the ease of accessibility via the patch electrode. Electrophysiology at this synapse has allowed a detailed examination of numerous properties of transmitter release. The RRP found at the calyx of Held is extremely heterogeneous in the synaptic vesicle probability of release. The main function of the calyx of Held is to faithfully propagate presynaptic signals even at high presynaptic firing frequencies. A critical characteristic, which permits the calyx to function as a relay synapse, is the large number of active zones. Vesicle release per active zone is maintained at levels that mimic other traditional presynaptic terminals, found throughout the brain. The calyx of Held undergoes a drastic developmental shift just prior to hearing onset (~P10). Around hearing onset there are numerous pre- and postsynaptic changes that occur which are required to allow the calyx of Held to function as a reliable relay synapse. These changes include a decrease in the release probability, changes in Ca²⁺ channel coupling, an increase in
the size of the RRP, an increase in quantal size, increased K+ channel conductance, faster gating of glutamate receptors and a shift from NMDA to mainly AMPA receptors.

The calyx of Held synapse plays a pivotal role in the mammalian auditory system. The main job of this synapse is to relay information about the location of sound with the use of fast and precise neurotransmission. The ability to faithfully propagate a presynaptic signal independent of presynaptic firing frequency is achieved by its highly specialized structure and enormous size. As discussed above, the calyx of Held undergoes anatomical, morphological and physiological changes throughout development (see Figure 2). The giant size of the calyx of Held allows for direct electrophysiological recordings, which is not possible in many areas of the brain that contain small conventional synapses (see Figure 3).

What allows the calyx of Held to reliably and faithfully transmit information under conditions of high firing frequencies? Changes that occur during development surely play a role, but given the distance from the nerve terminal to the cell body (~2 mm in mouse brain slices); could there be another mechanism? Under conditions of high metabolic stress or activity, there are a myriad of proteins that need to be dynamically regulated, at the nerve terminal to elicit an appropriate and precise response. We hypothesize that local presynaptic protein synthesis plays a role in maintaining this sensory synapse as a reliable information processor even under times of high metabolic activity. My thesis focuses on testing the physiological output of the calyx of Held nerve terminal under conditions of global protein
synthesis inhibition. The next section is going to discuss local translation in neurons, and the current state of the field.
CHAPTER 2

**Introduction to Local mRNA Translation**

The maintenance and dynamic regulation of the cellular proteome depends on protein synthesis. The mechanism behind protein synthesis involves the use of specialized RNAs (adaptor) and enzymes to translate the information encoded in mRNA (message) to a functional output, the protein. More than 50 years ago the “adaptors” and enzymes were elucidated to be (transfer RNAs) tRNAs and ribosomes, respectively. Over the past 20 years it has now been discovered that translation of protein from mRNA is regulated temporally, but also at a subcellular level spatially. Previously believed to only occur exclusively in the neuronal cell body, protein synthesis has been shown to occur in specific subcellular compartments far from the soma, a process called local translation. Neurons are some of the most structurally complex cell types located in the mammalian body. These unique cells exhibit a high degree of spatial compartmentalization, which provide the neuron with polarity. A fundamental characteristic of neurons is their ability to be excited with electrical stimuli. In order for a neuron to function as an electrically excitable cell it must receive, process, integrate, propagate and transmit information (Figure 4). A single neuron can receive signals at thousands of independent contact sites, or synapses. Interestingly the potency of the incoming signal can be regulated dynamically at single synaptic sites independent of other inputs. It is critical for neurons to function in a spatially restricted manner and execute fast and dynamic signaling with high fidelity. Taken together, the complex
functionality and morphology of neurons make local translation a vital process in regulating neuronal maintenance and physiology \(^{68}\).

Over the last 20 years or so it became clear that gene expression could also be controlled locally, with the use of messenger RNA (mRNA) targeting to certain subcellular addresses. Extrinsic signals are often concentrated to certain parts of the neuron to elicit a specific type of response. The use of RNA based localization mechanisms provides a bridge between the extrinsic signal seen at a certain subcellular location and the functional response from the appropriate part of the neuron. Localization of mRNA allows neurons to respond in a precise, rapid and dynamic fashion. A single message can give rise to a multitude of proteins in an extremely short period of time \(^{70}\). This is accomplished by several ribosomes (polysomes) binding the 5’ cap of an mRNA and producing several copies of a single protein off of one transcript. This suggests that local targeting of mRNA and subsequent local protein synthesis would be more efficient than protein transport under conditions when a large quantity of a certain protein is rapidly needed at a certain subcellular location \(^{71}\).

Local protein synthesis provides a method to locally sustain and transform the synaptic proteome. Localization of mRNA in dendrites and axons permits rapid synthesis of necessary proteins needed to carry out an appropriate response to diverse stimuli \(^{72}\). This also avoids problems that could arise with cargo transport, and cellular energy consumption (transport is not free). During development growth cones are steered by external cues that ultimately stimulate local translation
of cytoskeletal components or regulators. In order for a growth cone to properly turn toward an attractive guidance cue local translation of \( \beta\)-actin mRNA is required \(^{73}\). Conversely, in response to a repulsive cue the growth cone needs to disassemble actin filaments and push away, this is accomplished through the local production of \textit{cofilin} \(^{74,75}\). Another critical step in development during axon path finding is growth cone collapse. Local translation of \( \text{RhoA} \) mRNA is required for Semaphorin-3A mediated growth cone collapse \(^{74}\). There are a plethora of mRNA that have been discovered in dendrites of mature neurons as well \(^{76}\). The localization and regulated translation of these mRNAs allows for synapse-restricted responses to certain types of neuronal stimulation \(^{77}\). In summary, numerous studies in the field of local protein synthesis have demonstrated the importance of mRNA localization and subsequent local translation. This is an extremely dynamic and efficient means to regulate gene expression.
CHAPTER 2.1

Local Translation in Axons and Dendrites

There are two general criteria that have to be met to allow local protein synthesis: 1. The translational machinery and mRNA of interest have to be present in the source compartment (the site of synthesis) 2. The stimulus required to induce translation has to be detected and processed by the translational machinery. The intersection of these two criteria dictates the site of protein synthesis. A good starting point for determining if a neuronal sub-compartment has the capacity to perform local translation is to probe for the components required to execute protein synthesis. Identification of polyribosomes at the base of dendritic spines combined with metabolic labeling experiments provided the first piece of evidence that on the site protein production occurs in neurites. This was followed by the identification of specific mRNAs localized to synaptic sites. The positioning of various subsets of mRNAs has been coupled to synaptic plasticity mediated by local protein synthesis in dendrites. This provided highly plausible evidence that local translation occurs in the dendritic compartment. However, there was still a great deal of speculation in regards to translation occurring in axons. Most of the controversy was concerned with polyribosomes observed exclusively in growth cones and not in the mature axoplasm. Almost three decades later this was explained by the subcellular tethering of polyribosomes close to the plasma membrane, making it quite difficult to observe with ultrastructural analysis. A key piece of data that was highly suggestive of local translation being required for
Axonal function came from cue-induced directional steering being prevented with application of translational inhibitors, in surgically isolated axons. Subsequent studies have shown that local axonal translation is required for growth cone collapse. There are a number of studies that support local translation occurring in the axonal and dendritic compartments. Taken together, local translation in dendrites and axons is now an accepted dogma.
CHAPTER 2.2

**Biological Functions and Molecular Control of Local Translation**

In order for local protein synthesis to be possible several components need to be present: mRNA, ribosomes, transfer RNA (tRNA) and all the enzymatic components required to effectively execute translation. Translation of a protein spatially and temporally, at the locale of need, offers several advantages over transportation of a pre-existing peptide from one compartment in the neuron to another. This implements precision to protein localization, placing a protein only where it is needed and not anywhere else. Finally, newly translated proteins can replenish degraded, damaged or silenced proteins to maintain the homeostatic properties of the local proteome.

The positioning of mRNA at certain subcellular locations in the neuron enables a more rapid and dynamic response to environmental stimuli. Molecular control of translation permits accelerated changes in protein quantity. Control can be accomplished through multiple mechanisms such as changing the amounts and behaviors of protein synthesis components: ribosomes, translation factors and tRNAs. Spatial localization and regulation of translation is a discrete mode of gene expression that positions gene function with extreme precision in time and space.
CHAPTER 2.3

Local Translation and Human Disease

The translation of mRNA to protein plays a pivotal role in every cell type found in living organisms. Loss-of-function mutations in translation components usually result in defects restricted to the nervous system. The significance of elucidating presynaptic protein synthesis in vertebrates can be illustrated when discussing Fragile X. FMRP (Fragile X Mental Retardation Protein) is a negative regulator of translation. Mechanistically, it binds to the coding sequences of polysome-bound mRNAs and stalls elongation. Fragile X syndrome (FXS) is a genetic illness that is an inherited cause of intellectual disability. It is characterized by intellectual disability, disruptive and autistic-like behavior, epileptic seizures, language deficits and autism. FXS is caused by a mutation in the human FMRP-coding gene (Fmr1). Studies performed on the developing rodent brain detected FMRP-containing granules in dendrites and axons. Interestingly, loss of FMRP function results in defective formation of pre- and postsynaptic terminals. These studies indicate that unregulated protein synthesis caused by FMRP loss of function, results in the development of abnormal neuronal connections. This ultimately leads to the behavioral defects observed in FXS patients.
CHAPTER 2.4

Local Translation at the Presynapse

Long-lasting morphological changes, at the level of the synapse, depend on protein synthesis. A penetrating subset of this synthesis has been extensively researched in the postsynaptic compartment. What about the inevitable contribution of protein synthesis in the presynaptic compartment? Local presynaptic translation is a relatively new concept for neurobiologists, who a short time ago thought the central dogma of molecular biology (DNA->RNA->Protein) was carried out exclusively in the soma. The main objection to an exclusive somatic source of presynaptic protein production comes from the fact that cytosolic and structural proteins synthesized in the cell body reach the terminal via slow axoplasmic transport. The rate of this type of transport is approximately a few mm/day. Axons in the peripheral nervous system have the ability to extend distances of > 1m, thus it would require substantial time for some proteins to reach their presynaptic destination. The ability to respond to the cells metabolic requirements would not be realistic. There has been a good amount of experimental evidence, from several model systems, that has educated us on presynaptic translation. The initial piece of data was generated from intact rodent brains, where radiolabeled amino acids were rapidly incorporated into growing presynaptic polypeptides. These data are highly suggestive, however they do not assign a location to the site of protein production. The majority of evidence to support presynaptic protein synthesis has been generated in invertebrates, where
synapse formation and plasticity have been implicated. There have been a number of studies in vertebrate models that also suggest the occurrence of presynaptic protein synthesis. Work performed in rat hippocampal neurons revealed data that suggested local translation is critical for synaptic vesicle recycling. Recently, it has been shown in rodent hippocampal slices that presynaptic protein synthesis is required for long-term depression of GABA release. This involves cap-dependent translation in interneuron axons, but not the cell soma. This study ultimately indicated that eukaryotic ribosomes are present in inhibitory interneuron axons and terminals. Mounting evidence demonstrates local presynaptic translation, occurs in at least a subset of neurons. This raises a few fundamental questions: Presynaptic protein synthesis is required for long term depression in hippocampal interneurons, however does this process also occur in excitatory neurons? Does local translation only occur under times of plasticity? Is it restricted to only long-term forms of plasticity? To elucidate the answers to these questions a unique type of model synapse is needed.

To date there are no definitive studies from vertebrates that unambiguously prove that local translation occurs in an excitatory presynaptic compartment. Furthermore, it is important to understand if local translation occurs under non-stimulated conditions to maintain synapse homeostasis. A vertebrate model synapse is needed that allows researchers to separate pre- versus postsynaptic physiology. Furthermore, an ideal model synapse would be one in which the presynaptic compartment is amenable to manipulation and electrophysiological recordings. The calyx of Held is an intriguing model to address this phenomena, where the distance
between the soma and presynaptic terminal is \( \sim 2 \) mm, in rodents\(^{108}\). My thesis investigates local presynaptic protein synthesis using the murine calyx of Held synapse to determine if presynaptic translation occurs and is required for synaptic function.
CHAPTER 3

The Calyx of Held as a Model Synapse to Study Local Protein Synthesis

Rationale for using the calyx of Held to address the presence of local presynaptic protein synthesis and its requirements for certain aspects of synaptic transmission.

1) The calyx of Held allows dual pre- and postsynaptic patch-clamp recordings, which provide real time measurements of input-output relationships in response to translational inhibition (see Figure 3).

2) The calyx of Held synapse has been extensively studied and basic mechanisms of both pre- and postsynaptic physiology have been described in great detail.

3) This synapse is capable of firing at high frequencies for sustained periods of time. This permits driving this synapse to a point where replenishment of key proteins is required to maintain faithful synaptic transmission. Experiments performed on local translation in neurites show that production needs to be stimulated (basal versus stimulated) chemically or electrically.

4) The relationship between the calyx and its postsynaptic partner, the principal neuron located in the MNTB, is one to one (see Figure 3). This means that stimulation of a single axon induces release onto a single postsynaptic neuron. This allows us to easily interpret our findings, because our data won’t be confounded by additional axonal recruitment.

5) Recordings can be performed during a critical developmental period at this
synapse. Presynaptic recordings can be performed up to 5 days before and ~8 days after the onset of hearing (~P18), and postsynaptic measurements can be done at any time period after a functional synapse has been created.

6) Short-term plasticity has been well characterized and studied at this synapse, which allows us to assay synapse strengthening (post-tetanic potentiation PTP), monitor the probability of release (Pr) via paired pulse facilitation or depression, and measure changes in the readily releasable pool (RRP). Long-term plasticity has not been observed at this synapse.
CHAPTER 4

Shorthand Version of Introduction (Publication Version)

Coordination of ongoing synaptic transmission is fundamental to brain function, and requires the synthesis, localization, interaction and maintenance of thousands of pre- and postsynaptic proteins \(^{109}\). The identity, location, and stoichiometry of each protein must be tightly regulated in order to maintain precision and fidelity during signal propagation. Neurons are a unique cell type due to their highly structured and polar morphology. This places biological constraints on neurons due to information processing that occurs in remote locations (axons and dendrites) \(^{110}\). Synaptic proteins are typically thought to be synthesized in the soma and transported to synapses, but several groups have demonstrated that some postsynaptic proteins can also be locally synthesized in dendrites \(^{78,81,84,111}\), and local synthesis is required for some forms of synaptic plasticity \(^{84}\). Over the past decade RNA based mechanisms have been uncovered as a link between extrinsic signals and the operative state of the neuron \(^{71,72,95}\). This is possible due to the targeting of coding and non-coding RNA, RNA binding proteins, as well as ribosomes to specific subcellular compartments. This provides the neuron with all the necessary components in place to translate particular proteins, on-site, when induced by a specific signal. It remains unclear to date how and if local translation is required for standard synaptic transmission, as well as periods of sustained neurotransmitter release.

Local protein synthesis is thought to provide a faster and more efficient mechanism for dendrites to maintain normal activity levels and respond to rapidly
changing inputs \(^{112}\). Although local protein synthesis at or near the nerve terminal has been shown to occur in several animals \(^{102}\), evidence for presynaptic protein synthesis in the mammalian brain has been difficult to demonstrate, largely due to the difficulties of specifically accessing presynaptic terminals. Despite these difficulties, presynaptic protein synthesis has recently been shown to occur in GABA-ergic interneurons in hippocampus of mice, where it is necessary for long-term depression of synaptic responses \(^{107}\). Therefore, local protein synthesis can occur at both sides of the synapse in mammalian brains, and it is necessary for some aspects of synaptic transmission.

Local presynaptic protein synthesis would provide the neuron with the ability to dynamically respond to confined synaptic activity \(^{113}\). This would allow neuronal signals to be processed independent of the cell body and axonal transport mechanisms. This is important due to axons having the ability to extend several centimeters in vertebrates, and tens of micrometers when viewing local neuronal circuitry \(^{113}\). In addition, the rate of axoplastic transport has been reported at 1-4 mm/day with protein half-lives of 1-2 weeks for cytoskeletal proteins \(^{113}\). If protein transport were the only source of presynaptic proteins, then nerve terminals couldn’t extend beyond a few centimeters.

Activity in postsynaptic neurons has been shown to increase local protein synthesis \(^{107,114}\). The rate of protein synthesis that is required to replenish the proteins that are necessary to maintain ongoing synaptic activity is not fully understood. While some proteins are stable for days, others have a much shorter
half-life \textsuperscript{115}. We reasoned that the relatively high basal and maximal firing frequency of neurons in the auditory brainstem \textsuperscript{116} could require a high level of protein synthesis and may also require periods of rapid local protein synthesis to maintain activity. To better understand the role of protein synthesis in highly active neurons, we have used the calyx of Held synapse, located in the medial nucleus of the trapezoid body (MNTB) in the auditory brainstem \textsuperscript{1}. This synapse is involved in sound localization, and can fire at up to 1 KHz for short durations \textsuperscript{117}. The basal firing rate is $\sim$20 to 50 Hz \textsuperscript{117}, and it can fire for longer durations at frequencies of 100 to 200 Hz. The calyx of Held is a large, glutamatergic nerve terminal that forms a monosynaptic; axosomatic connection to MNTB principle cells (Figure 1 and Figure 3). This large presynaptic terminal contains hundreds of individual release sites, and the size of the terminal facilitates imaging and presynaptic patch clamp recordings (Figure 3). In addition, the basic mechanisms of pre- and postsynaptic responses have been extensively characterized at this synapse \textsuperscript{15,118}. This synapse also undergoes significant developmental changes in its morphology and physiological characteristics that occur around the onset of hearing in mice, at approximately postnatal day 10 \textsuperscript{15}. Finally, in a mouse brain, the calyx of Held nerve terminal is over 2 mm away from the cell body (Figure 1). This distance could facilitate the need for local translation at the nerve terminal. These characteristics make this synapse an excellent choice for studying the effects of protein synthesis on ongoing synaptic activity.

Our data indicate that ribosomal components are present in the calyx of Held nerve terminal under basal conditions. We have also found that presynaptic
ribosomes are present and fully functional under non-stimulus conditions. In addition, we show that inhibiting protein synthesis at this synapse causes an increase in the frequency and speed of spontaneous neurotransmitter release. We also find that control and translationally inhibited neurons normalize to the same spontaneous release rates following tetanic stimulation. Moreover, the paired pulse ratio is affected under conditions of inhibiting protein synthesis. These findings strongly indicate that local presynaptic protein synthesis can occur at the calyx of Held nerve terminal, and our functional data indicate a presynaptic mechanism of action.
CHAPTER 5

Materials and Methods

Slice Preparation and Electrophysiology

Brain slices. C57BL6 mice (Charles River Laboratories) from postnatal day 8 to 12, of either sex were used for all experiments described. The mice were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, and protocols used for handling and care were reviewed by the Rutgers University Animal Care and Facilities Committee. Animals were decapitated without prior anesthesia, in accordance with NIH guidelines. Transverse brainstem slice thickness varied from 100 µm (immunohistochemistry and imaging) to 180 µm (electrophysiology) and were generated using a Leica VT1200 vibratome. Throughout the process of dissection and slicing, the brain was maintained in a low-calcium artificial CSF (aCSF) solution at 1-2°C containing the following (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 25 glucose, 0.8 ascorbic acid, 3 myo-inositol, 2 Na-pyruvate, 3 MgCl₂, and 0.1 CaCl₂, pH 7.4, when oxygenated with carbogen gas (95% oxygen, 5% carbon dioxide). Once produced, slices were transferred to a holding chamber maintained at ~35°C for 30-40 min in normal calcium aCSF solution with the same composition listed above except for 1mM MgCl₂ and 2mM CaCl₂. This same solution was also used as the standard recording solution for electrophysiology experiments (see below). All experiments were performed at room temperature (22-25°C) for up to ~4-5 hours after the recovery period.
*Electrophysiology.* Patch-clamp recordings were conducted using an EPC10 USB double patch-clamp amplifier with PatchMaster software (HEKA; Harvard Bioscience). A transverse slice orientation was used in all postsynaptic voltage-clamp recordings in order to maintain the integrity of the calyceal axons for fiber stimulation. Patch pipettes were produced from thick-walled borosilicate glass, 2.0 mm outer diameter, 1.16 mm inner diameter (Sutter Instruments). Postsynaptic pipettes (2-3 MΩ) were filled with a solution containing (in mM): 125 Cs-methanesulfonate, 20 CsCl, 20 TEA, 10 HEPES, 5 phosphocreatine (Alpha Aesar), 4 ATP, 0.3 GTP, and 2 QX-314 Cl⁻ (Sigma Aldrich; to block voltage gated Na⁺ channels on the postsynaptic neuron to measure the true EPSC), and was buffered to pH 7.4 using CsOH. For all electrophysiology recordings and data analysis, drug treatment and controls were blinded to the experimenter. To establish the quality of the slices, neurons, and general recording conditions, 1-2 initial recordings were obtained each day in normal aCSF, supplemented with 25mM bicuculline and 2mM strychnine (Figure 5). If the initial recordings had stable responses that lasted the duration of the stimulus protocols, a minimum of 25 minutes, then the recording solution was switched to a blinded cylinder for subsequent recordings which were performed after ~ 1 hour of treatment (45 minutes to 120 minutes). On each day, the blinded cylinder would contain either: 40μM anisomycin (Sigma Aldrich) or DMSO alone (vehicle). Postsynaptic series resistances (Rₛ) for voltage-clamp recordings were typically 4-10 MΩ. An Rₛ compensation of 75-80% was applied for all recordings such that the adjusted Rₛ were in the range of 2-5 MΩ. Cells that did not meet these criteria were excluded from analysis. Currents were filtered by a 4-
pole Bessel filter at 3kHz to remove residual high-frequency noise present in the recordings. Holding potentials were set to -65mV. Junction potentials, calculated to be -11 mV, were not corrected.

Calyx synapses in the medial nucleus of the trapezoid body (MNTB) were afferently stimulated (A-M Systems Isolated Pulse Stimulator Model 2100) using a bipolar fiber stimulator (lab design) placed at the midline of the slice. The MNTB field was scanned with an extracellular pipette to locate neurons that respond to midline fiber stimulation. (See Figure 6 for detailed stimulation flow diagram)

To test the effect of the translational inhibitor anisomycin (40 µM) on synaptic response characteristics, slices were preincubated for ~1 hour in the presence of the drug in the absence of afferent fiber stimulation. At all times, aCSF was continuously circulated using a peristaltic pump; total volume of the solution was 30mL.

In order to determine the physiological consequence of translational inhibition, whole-cell postsynaptic patch clamp recordings were performed on brain slices from (P8-12) animals. All experiments were double-blinded in data acquisition and analysis. Briefly slices were perfused with normal artificial cerebral spinal fluid (aCSF) and calyceal axons were stimulated using a bipolar afferent fiber stimulator in order to elicit a postsynaptic response (Figure 5). Control recordings were performed prior to bath application of the translational inhibitor anisomycin, or the vehicle control (DMSO). To fully investigate the affect of translational inhibition on synaptic transmission, a very detailed stimulus paradigm was
developed (Figure 6). Only neurons where I was able to complete the entire stimulus protocol were included for analysis. The paradigm is designed in a way that allows us to assess different properties of synaptic communication at this synapse with a single recording.

*Recordings from MNTB neurons:* All recordings were made in the presence of 25µM bicuculline and 2µM strychnine, at a holding potential of -65mV. Recording solution and pipette solution are the same as described above. Miniature excitatory post-synaptic currents (mEPSCs) were recorded during 30 sec continuous recordings at several times during the stimulation protocol. Spontaneous events were analyzed with Mini Analysis Software (Synaptosoft). The following mEPSC search parameters were used: gain, 20; blocks, 3,940; threshold, 10 pA; period to search for a local maximum, 20,000 ms; time before a peak for baseline, 5,000 ms; period to search a decay time, 5,000; fraction of peak to find a decay time, 0.5; period to average a baseline, 2,000 ms; area threshold, 10; number of points to average for peak, 3; direction of peak, negative). These parameters were provided by a recent publication that studied spontaneous release at the calyx of Held. Analysis was initially performed by the software, and then visually checked to ensure accuracy.

**Statistical Analysis**

Data are presented as mean ± s.e.m. All data shown are paired data and
unless noted a Student’s t-test was employed. Statistical significance was considered when p-value was ≤ 0.05 and significance level is denoted using asterisks (*p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001).

The two-sample Kolmogorov-Smirnov (KS) test was used to calculate the p-value for the cumulative probabilities of the mEPSC event intervals for two different conditions. Briefly, this non-parametric test uses the maximum vertical difference between two cumulative probability graphs and the total number of measurements to determine the statistical significance (given by the p-value) of the differences between two cumulative probability distributions. Since the exact durations of the mEPSC intervals cannot be identical between two different conditions, it is necessary to produce histograms with identical bin-values to compare the mEPSC intervals for the two different conditions being compared. We performed this calculation manually, and then we compared our calculated values to values generated by the KS function in MATLAB, and found very similar or identical values. The small differences that were sometimes found between our calculations and MATLAB were due to differences in the size of the bins used to generate the histograms. To be conservative in our calculation of the p-value, we used the total number of non-zero bins in the histogram as our n-value for the total number of measurements.

**Immunohistochemistry and Confocal Microscopy**
**Immunohistochemistry:** Either sex of C57BL6 mice (postnatal (P) day 8 to 12, n=18) were decapitated without previous use of anesthesia, and transverse auditory brainstem slices (100-140 mm thick) were prepared as described above. Following recovery in normal aCSF, sections were transferred to a 12 well culture plate and washed 2x in phosphate buffered saline (PBS) (in mM: 137 NaCl, 2.7 KCl, 4.3 Na$_2$HPO$_4$ *7H$_2$O, and 1.4 KH$_2$PO$_4$, pH 7.4). Following the washes, the solution was replaced with ice-cold PBS containing 4% (wt/vol) PFA and fixed for 30 min at room temperature, with gentle agitation. After fixation the sections were rinsed 3x with PBS, and incubated in blocking and permeabilization buffer in PBS containing 10% (vol/vol) normal goat serum (MP Biomedicals, LLC), 2% (wt/vol) BSA and 0.25% (vol/vol) Triton X-100 (Alfa Aesar) for 1h30m at room temperature. Slices were again rinsed with PBS 3x, 10m each wash. Sections were further blocked in PBS containing 40mg/mL of AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) for 1 hour at room temperature. Slices were then washed (3x) and placed in PBS containing the following; 1% (vol/vol) normal goat serum, 1% (vol/vol) BSA, 0.25% (vol/vol) Triton X-100, and mouse monoclonal anti-5.8S rRNA, clone Y10b at 1:500 (Abcam, ab37144) overnight at 4°C. Importantly, to minimize the possibility of non-specific interactions, all double labeling experiments were done sequentially. Following overnight incubation, slices were washed 3x in PBS, and placed in primary antibody solution containing guinea pig polyclonal anti-vesicular glutamate transporter 1 (VGLUT1) at 1:500 (Synaptic Systems) and allowed to incubate overnight at 4°C. Slices were rinsed 3x in PBS, and placed in PBS containing the following; 1% (wt/vol) BSA, 0.05% (vol/vol) Tween-20, and Alexa-594-conjugated
AffiniPure Goat Anti-Mouse IgG (H=L)(1:500) secondary antibody (Jackson, 115-585-003) for 2h at room temperature. Slices were rinsed 3x in PBS and then incubated in the same buffer as above, but with Alexa-488 conjugated AffiniPure Donkey Anti-Guinea Pig IgG (H+L)(1:500) secondary antibody (Jackson, 706-545-148) for 2h at room temperature. Sections were then washed with PBS and mounted to a glass slide, excess PBS was removed, a few drops of Fluoromount™ (Sigma Aldrich) was added, and covered with Gold Seal cover slips #1.5 (Thermo Fisher). Slides were stored at 4°C.

To confirm 5.8S rRNA specificity we pretreated slices with nucleases. Following fixation sections were washed in PBS and incubated in PBS containing 0.25% (vol/vol) Triton –X100 for 45 minutes. Slices were then washed 3x (10m each) in enzyme buffer (50 mM Tris and 5 mM CaCl₂, pH=8). Next, slices were incubated in enzyme buffer containing 80 mg/mL RNase A (Fermentas, EN0531) and 300 U/mL micrococcal nuclease (New England Biolabs, M0247) for 60 min at 37°C. (Note: control experiments were performed by incubating slices in enzyme buffer (with no enzymes) at 37°C) Following incubations, slices were rinsed 3x (10m each) with PBS and blocked for 1h30m in PBS containing 10% (vol/vol) normal goat serum and 2% (wt/vol) BSA, at room temperature. Antibody application and further slice processing is the same as described above.

Confocal Microscopy and Image Analysis: Confocal image stacks of PFA treated brain slices were acquired using an upright Leica TCS SP2 UV-equipped laser-scanning confocal microscope (Leica, Heidelberg, Germany). Image acquisition was
performed in sequential scanning mode with non-overlapping photomultiplier tube settings. All data were acquired with the pinhole set to 1 airy unit which corresponds to Z-stack optical sections of ~300 nm. Z-stacks were collected with a depth difference of ~0.3 µm between each Z section. High-resolution image stacks were imported into FIJI. Regions of interest (ROI) were used to calculated image intensity of individual presynaptic terminals, postsynaptic principal cells, or both. Co-localization was performed by selecting neurons from the same-stacked images at random. These marked cells were numbered to prevent and neurons being counted in duplicate. The selected cells were cropped into a separate image. Analysis was performed on the Z-section where the neuron was widest in order to reduce false positive signals as a consequence of bleed through. Next the wand tool was used to select the appropriate compartment to measure co-localization (presynaptic, postsynaptic, or both). Once the “target” membrane is selected we then cleared the outside of any extraneous signal. To further ensure analysis is not performed on any non-specific or non-neuronal signal we background subtracted each image (a value of 10) for all channels being tested. To perform the co-localization analysis the FIJI plugin JACop\textsuperscript{120} (just another co-localization plugin) was used. This generated a graphical output table that contained the Pearson's correlation coefficient.

Line scan analysis to qualitatively assess signal overlap in a given area was performed in FIJI using the plot profile feature. Relative pixel intensity values were exported to excel. In all experiments performed the VGLUT1 signal was always
higher in relative intensity than 5.8S rRNA and puromycin. To pseudo-normalize this difference, the lower intensity signals were plotted on a secondary y-axis.

SUnSET Labeling of Newly Synthesized Proteins

*Puromycylation Assay:* Transverse brainstem slices were prepared as described above. To detect newly synthesized proteins, slices were subjected to puromycin (1.8 μM Sigma Aldrich) added to normal aCSF following the brain slice recovery period, for 15 min. Control slices were pre-incubated first with 40 μM anisomycin for 60 min, and puromycin was added at the 50 min time point so that anisomycin and puromycin were present for the last ten minutes. Following incubation slices were rinsed 3x with pre-warmed PBS, prior to fixation. The slices were then further processed as described above, post-fixation. To detect purompsych peptide a mouse monoclonal anti-puromycin antibody, clone 12D10 (EMD Millipore, MABE343) was used at a dilution of 1:250. The secondary antibody used for indirect immunofluorescence was Alexa-594-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) at a dilution of 1:500. Imaging was carried out as described above.
Chapter 6

Evidence for presynaptic ribosomes at the calyx of Held nerve terminal

The calyx of Held is a large glutamatergic, monosynaptic nerve terminal located in the medial nucleus of the trapezoid body (MNTB) in the mammalian auditory brainstem (Figure 1 and Figure 3). In addition, the axon projects a significant distance from the cell bodies in the anterior ventral cochlear nucleus to the MNTB, which is >2 mm in young mice (Figure 1). Early in development, up to approximately postnatal (P) 12, the calyx has a spherical or spoon shaped morphology (Figure 7, top). This large spherical morphology that is prevalent up to P12 and provides a clear image of the presynaptic compartment, allowing the ability to distinguish fluorescent signals in the presynaptic terminal and the postsynaptic soma. After P12, the calyx terminal begins a change to a fenestrated morphology that is prevalent by P16, and is considered to be mature in morphology and function (Figure 7, bottom). The morphological changes are accompanied by changes in protein expression that allow faster action potential kinetics and synaptic release properties that begin at ~P10, and allow this synapse to function at the high frequency and fidelity that is required for sound localization. Accordingly, there is a high level of protein turnover that occurs slightly before and continues throughout this period.

We hypothesized that local protein synthesis could occur at this nerve terminal, particularly due to the long axon length, the large size and the high frequency firing that requires high levels of protein to maintain activity at >600 individual release sites in a calyx nerve terminal. To investigate if local protein synthesis occurs in
the calyx of Held, we used imaging techniques to determine if a major ribosomal component that is necessary for protein synthesis is present in the presynaptic terminal. The presence of ribosomal components is a very strong indicator of the presence of ribosomes and local protein synthesis. A major and necessary component of eukaryotic ribosomes is the 5.8S ribosomal RNA (rRNA) which is required to execute ribosomal translocation \(^{128-130}\). This component has previously been shown to be present in dendritic compartments, and provides evidence for ribosomes and local protein synthesis in dendrites \(^{76,111,131,132}\). Recent work has shown that 5.8S rRNA is present in CA1 inhibitory interneuron nerve terminals \(^{107}\), and is also present in axons \(^{129,133-135}\) and neurites. However, given the small size of these compartments, standard-imaging techniques can be difficult. Therefore, the large size of the calyx of Held helps to better determine the presence and localization of 5.8S rRNA in the nerve terminal.

Immunolabelling for 5.8S rRNA in brain slices shows a robust signal (Figure 8 A, B), particularly in neuronal somata, consistent with the high levels of protein synthesis that occur in the cell body \(^{136-138}\). To label the large calyx of Held nerve terminal, an antibody against the vesicular glutamate transporter, VGLUT1, was used (Figure 7, 8 A, B and Figure 9 A) which is the standard approach to specifically label this presynaptic terminal \(^{123,139-142}\). We typically observe several areas that exhibit 5.8S rRNA labeling in the presynaptic terminal, as shown in the representative images (Figure 8 A, B). The average intensity ratio for the presynaptic terminal to background signal is 3:1, which allowed us to clearly distinguish the presynaptic signal. As expected, the average intensity for the signal that was present in the
presynaptic terminal was less than that of the somata with an average intensity ratio of 4:1 for the ratio of soma to presynaptic signals. Despite the strong postsynaptic fluorescence, we were able to unambiguously identify numerous areas with clear presynaptic signals (see white arrows in Figure 8 A, B). To further demonstrate the overlap of the 5.8S signal with the VGLUT1 signal, we performed line scan analysis (Figure 8 C) to compare the positional overlap of the VGLUT1 and 5.8S rRNA intensities at higher magnification (Figure 8 B; line scan shown in right panel). We find a high correlation between the relative intensities of the 5.8S rRNA and VGLUT1 signals (Figure 8 C, merge). Interestingly, we note peaks and troughs in the intensity of the VGLUT1 signal are often replicated in the intensity of the 5.8S rRNA signal, which is expected given the presence of organelles and other presynaptic components that would act to reduce both signals. Finally, we performed correlation-coefficient analysis to quantify the overlap between VGLUT1 and 5.8S rRNA. We calculated a Pearson's correlation-coefficient (r) of (0.78±0.04 s.e.m.) for VGLUT1 and 5.8S rRNA signal in the presynaptic terminal. In comparison, we calculated a Pearson's r-value of (0.11±0.01 s.e.m.) in the region surrounded by the VGLUT1 signal, which primarily indicates the somatic compartment of the postsynaptic neuron (Figure 8 D, n=13 neuronal pairs, p<0.001). These data strongly indicate that ribosomes are present in the calyx of Held presynaptic nerve terminal.

To verify the specificity of our 5.8S rRNA signal, we treated the brain slices with nucleases to degrade the 5.8S rRNA prior to antibody labeling. As expected, nuclease treatment eliminated the presynaptic and postsynaptic 5.8S
signal (Figure 9 A, center). To better visualize the location of any residual 5.8S signal that may have remained after nuclease treatment, we increased the signal contrast (Figure 9 A, right panel), which still shows a complete lack of 5.8S signal in nerve terminals and somata. The average pixel intensity for the combined pre- and postsynaptic compartments in untreated slices (68.30±7.33 s.e.m., n=10) was far greater than the low remaining signal in nuclease treated slices from the same brain (0.12±0.01 s.e.m., n=10). These data further indicate the presence of ribosomes in the presynaptic terminal, suggesting the ability for local protein synthesis at this nerve terminal.
Chapter 7

**Functional presynaptic ribosomes**

Our data show that ribosomal components are present in the presynaptic terminal. To demonstrate if the ribosomal components are fully assembled and functional we used the surface sensing of translation (SUnSET) technique (Figure 10143). This technique allows us to directly visualize the general location of protein synthesis using a fluorescent signal that is proportional to the amount of translation. Briefly, this method uses puromycin, which mimics tRNA (Figure 10 A) and becomes incorporated into nascent polypeptide chains (Figure 10 B). Specific antibody labeling is then used to detect the amount and location of puromycylation events 144–146. As described below, our results using this technique confirm that functional ribosomes are present in the presynaptic terminal.

Consistent with studies in neuronal culture experiments 147, we found that a brief, 10 minute application of puromycin allowed us to detect ribosome activity. We found fluorescent signal in calyx of Held nerve terminals and in principle cell somata, with relatively low background activity from other nearby cells (Figure 11 A, C, E and Figure 12). To quantify the presence of active ribosomes in the presynaptic compartment, we calculated the Pearson’s correlation coefficient (r) for the puromycin and VGLUT11 signals. We find r-values of (0.74±0.05 s.e.m.) for the presynaptic terminal compared to (0.14 ± 0.03 s.e.m.) for the somatic region surrounded by the calyx nerve terminal (Figure 11 B, n=13 neurons, p<0.001). This further verifies that the necessary components for protein synthesis, including the
5.8S rRNA shown in figure 8, are present in the presynaptic terminal and are capable of forming translationally competent ribosomes.

To validate that the SUnSET assay is detecting active translation, we treated brain slices with the translational inhibitor anisomycin for 1 hour prior to the addition of puromycin (Figure 11 C). If ribosomes must be active and puromycin needs to be incorporated into a polypeptide chain to be detected by the SUnSET assay, inhibiting translation will greatly reduce or inhibit the puromycin signal. Consistent with this, we find that anisomycin treatment effectively eliminates the puromycin signal (Figure 11 C, center), giving a >100 fold reduction in the fluorescence intensity following anisomycin treatment (Figure 11 D). These results verify that the SUnSET assay provides an efficient and specific measurement of the presence and general location of active ribosomes.

Localizing puromycin fluorescence to the presynaptic terminal demonstrates the presence of active presynaptic ribosomes. As expected, the fluorescence intensity is higher in somata than in the terminal. At a higher magnification, puromycin labeling is clearly present in the presynaptic terminal demonstrating the presence of functional presynaptic ribosomes (Figure 11 E and Figure 12). To better visualize the relationship between puromycin labeling and the presynaptic marker VGLUT11; we used line scans to assess the degree of colocalization (Figure 11 E and Figure 12, bottom panels). We find excellent agreement in the location and relative intensity of the two signals, thus demonstrating that translationally competent ribosomes are found in the calyx of Held presynaptic nerve terminal. Furthermore,
all of the necessary components (mRNA, tRNA, rRNA, ribosomal proteins and ribosomal binding proteins), which are required to execute translation, must also be located in the nerve terminal.
Chapter 8

**Spontaneous synaptic events indicate presynaptic effects of inhibiting translation**

Spontaneous release events are sensitive to presynaptic release properties, which primarily affect the event frequency and postsynaptic changes in ionotropic receptor responses which affect the amplitude and kinetics of the responses \(^{148}\). Since the need for protein synthesis could be affected by prior activity, it was important to measure spontaneous activity at several times during our recordings to determine if inhibiting translation affects initial miniature excitatory postsynaptic currents (mEPSCs), and if mEPSCs are affected after evoked activity.

We find that the initial mEPSC frequency, measured shortly after break-in, is higher in cells treated with the protein synthesis inhibitor anisomycin (2.4 ± 0.7 Hz, \(n=11\) recordings) compared to untreated neurons (1.4 ± 0.3 Hz, \(n=10\) recordings) from the same animals (Figure 13 A, B). The initial cumulative probability histogram of the time between mEPSC events clearly shows that inhibiting protein synthesis decreases the time between mEPSC events compared to control recordings of neurons in untreated slices from the same animals (Figure 13 C, \(p<0.0001\), Kolmogorov-Smirnov test). For example, in neurons treated with anisomycin, 70 percent of the events occur with an interval less than ~350 msec (Figure 13 C, red dotted line), compared to ~700 msec for control recordings (Figure 13 C, black dotted line). To further examine this, we fit exponential curves to the cumulative probability data to better understand the time course of spontaneous release. We
find that both the control and the protein synthesis inhibited distributions are well fit by double exponential curves, indicating a fast component and slower component of spontaneous release (Figure 13 C, grey lines through data points). Both the control and anisomycin treated neurons have a fast component at approximately 170 msec, and a slower component which is 4 to 5 fold longer (Figure 13 C). Interestingly, the fast component accounts for the majority of mEPSC events for anisomycin treated neurons (64%), in contrast to control neurons where the fast component only accounted for 37% of the mEPSC event intervals. Thus, the percentage of fast versus slow spontaneous release events were equal but opposite in their distribution, demonstrating a similarity in fast and slow time components but a major difference in the percentage of fast versus slow release events. Therefore, inhibiting protein synthesis increases the prevalence of fast spontaneous release events.

In contrast to the differences seen in the frequency of mEPSCs, the amplitudes of mEPSCs were similar for control (36.8 ± 2.5 pA, n=10) and protein synthesis inhibited neurons (38.7± 2.0 pA, n=11), as shown in the cumulative probability of the mEPSC amplitudes (Figure 14 A), the amplitude histogram (Figure 14 A, inset) as well as the average amplitude graph (Figure 14 B). In addition, compared to controls, the protein synthesis inhibited neurons have a slightly faster rise time (Figure 14 C, p=0.19, left side of graph), and a slightly faster decay time (Figure 14 C, p=0.14, right side of graph), but these differences were not statistically significant. Consistent with this, the mEPSC area was slightly smaller for protein synthesis inhibited neurons compared to control neurons, but this difference was
also not statistically significant (Figure 14 D, p=0.16). The finding that the initial mEPSC frequency is increased after inhibiting protein synthesis, demonstrates a presynaptic effect on the probability of spontaneous release. The absence of effect on the mEPSC amplitude, and small effects on mEPSC kinetics suggests that the postsynaptic response is not significantly affected (within ~1 hour) by inhibiting protein synthesis.
Chapter 9

**Kinetics of evoked synaptic responses are relatively unaffected after inhibiting protein synthesis**

The finding that spontaneous release of synaptic vesicles is affected by inhibiting protein synthesis suggests that evoked responses could also be affected. In our initial tests, we stimulated at a low frequency (0.1 Hz) immediately following the initial mEPSC measurements to determine if the kinetics, peak amplitude and latency of evoked excitatory postsynaptic currents (EPSCs) are altered when protein synthesis is inhibited. Consistent with the results from the mEPSC experiments, the kinetics of the EPSCs appear to be unaffected by inhibiting protein synthesis over the time course of 45 to 120 minutes of inhibition (Figure 15 A). The 10-90% rise and decay times were similar, with small differences that were not significant (Figure 15 A, B, C). In addition, the average peak amplitude of the initial responses were also similar for control (5.7 ± 0.51 nA) and protein synthesis inhibited neurons (5.37 ± 0.73 nA), with no statistically significant difference between the amplitudes (Figure 15 D, p=0.6). Consistent with this, the average area of the initial EPSCs were also relatively unaffected between treated and control neurons (Figure 15 E, p=0.23). Finally, we also measured the latency of the EPSC responses and also found similar values for control and treated neurons (Figure 15 F, p=0.27). Taken together, these data suggest that the kinetics and peak amplitude of the initial evoked responses are relatively unaffected by inhibiting translation over an approximately 1 to 2 hour time course, which is similar to our finding that mEPSC kinetics and
amplitude are not affected by inhibiting translation. However, given the change in spontaneous release events, we anticipated a change in evoked release events, which was not apparent when we measured individual EPSCs. To further pursue this, we next tested if inhibiting protein synthesis affects high frequency EPSC responses.
Chapter 10

**Paired pulse measurements indicate a presynaptic effect of translational inhibition**

The calyx of Held can fire at high frequencies with high levels of precision. We hypothesized that presynaptic protein synthesis may play a role in presynaptic mechanisms of synaptic transmission. Although the kinetics and amplitude of initial responses do not appear to be affected (Figure 15), we hypothesized that high frequency synaptic stimulation may reveal differences in the responses. Therefore, we tested short stimulus trains, 400 msec at 200Hz followed two or more minutes later by a stimulus at 100 Hz. At both frequencies, we observed a tendency for lower levels of depression in the responses from translation-inhibited neurons. To quantify this, we measured paired pulse ratios, the ratio of the second response to the first response (P2/P1), during the two different trains with an interval of 5 msec (Figure 16 A1 and A2) or 10 msec (Figure 17 B1 and B2). Consistent with our preliminary observations, we find paired pulse depression in control cells at a pulse interval of 5 msec (0.86 ± 0.08 s.e.m., n=17 cells; Figure 16 A1 and A2), but a lack of depression in translation inhibited cells at the same interval (1.05 ± 0.07 s.e.m., n=12 cells; Figure 16 A1 and A2). Although this difference is not statistically significant (p=0.11), it suggests a partial facilitating effect. Interestingly, for the 100 Hz train, the 10 msec interval produced a statistically significant difference in the paired pulse ratio between the control cell responses (0.63 ± 0.05 s.e.m., n=17 cells; Figure 17 B1 and B2), and the response from translation inhibited cells (0.83 ± 0.06
s.e.m., n=13 cells; p=0.014; Figure 17 B₁, B₂). These differences in the paired pulse ratios are consistent with differences in presynaptic mechanisms involving vesicle release \(^{118,149}\), such that the translation inhibited cells are more resistant to depression.

Next, we hypothesized that ongoing translation may be necessary to maintain synaptic responses following excessive activity. Increased levels of synaptic transmission require increased rates of activity to restore ion gradients, vesicle availability, and other factors which may reveal effects on synaptic transmission that are not present at low activity levels. In addition, previous studies have shown that increased levels of synaptic activity initiate local protein synthesis in neurons \(^{150}\). Accordingly, we delivered two rounds of tetanic stimulation, each round consisting of 100 Hz for 4 seconds \(^{140,151}\), to produce a high level of prolonged synaptic activity. Following two rounds of tetanic stimulation, the paired pulse ratio at 5 msec decreased for untreated cells (0.67 ± 0.04 s.e.m., n=15 cells; Figure 16 A₃, control) but was only slightly reduced in cells treated with translational inhibitor (0.97 ± 0.06 s.e.m.; n= 10 cells; Figure 16 A₃, anisomycin), resulting in a highly significant difference (p=0.0002) that was still present >5 minutes later (Figure 16 A₄). This indicates that the resistance to paired pulse depression induced by inhibiting protein synthesis is stable, and that enhanced depression following prolonged activity in control neurons is slow to recover. Interestingly, there was very little change in the initial 10 msec paired pulse ratio (Figure 17 B₂) compared to the ratio following high activity for control (0.60 ± 0.05 s.e.m.; n= 16 cells; Figure 17 B₃, control) and translation inhibited cells (0.80 ± 0.04 s.e.m.; n= 12 cells; Figure
17 B3, anisomycin). This effect was stable throughout the recording, as indicated by paired pulse ratios measured >5 minutes after the tetanus trains (Figure 17 B4). Therefore, activity increases the 5 msec paired pulse depression under control conditions, but not when translation is inhibited. In contrast, paired pulse depression at a 10 msec interval is not further enhanced by activity in control and protein synthesis inhibited neurons. However, at both intervals, independent of activity, inhibiting translation produces a lower level of paired pulse depression, which strongly suggests an effect of translation on presynaptic activity. We conclude that inhibiting translation makes neurons less susceptible to paired pulse depression.

To determine if the reduced depression continues with repeated high frequency stimuli, we compared the peak amplitudes in the 200 Hz stimulation train up to the onset of the steady state response (Figure 18 A, B, C). This demonstrates that translation inhibited cells maintain far lower levels of depression compared to responses from control cells, even during repeated stimulation (Figure 18 C). The finding that inhibiting protein synthesis reduces depression even after the response has reached steady state levels indicates that the resistance to depression is robust during the time course of these recordings. While it is completely possible, and perhaps likely, that the resistance to depression will change with repeated activity over longer time periods, we were unable to reliably measure this given the need to maintain consistent control responses over longer time periods. We therefore stress that the enhanced synaptic response may be an initial consequence of inhibiting protein synthesis.
Chapter 11

**Enhanced spontaneous release following tetanus normalizes mEPSC frequency differences between control and protein synthesis inhibited neurons**

Tetanic stimulation produces a transient elevation in the frequency of spontaneous release events \(^{140}\). Given that inhibiting protein synthesis also increases the frequency of spontaneous release events, we decided to determine if the two effects are separate. Accordingly, we measured the frequency of spontaneous release before and after tetanic stimulation in control and protein synthesis inhibited neurons. Prior to delivering the tetanic stimulation, the control mEPSC frequency average was 2.5 ± 0.45 Hz, compared to 3.6 ± 0.89 Hz in protein synthesis inhibited neurons (Figure 19 A, A\(_1\)). It is important to note that the mEPSC frequency prior to the tetanic stimulation, for both anisomycin treated and control neurons, is higher than the initial mEPSC frequency measured at the beginning of the recordings (Figure 13). This is most likely due to stimulation trains delivered earlier in the protocol, consistent with evoked activity acting to elevate spontaneous release levels. The difference in the timing of mEPSC events in control and protein synthesis inhibited neurons prior to tetanic stimulation is statistically significant, and clearly visible in the cumulative probability histogram of mEPSC event intervals (Figure 19 A\(_2\); p<0.0001, Kolmogorov-Smirnov test). There is also a similar trend in the exponential fit of the cumulative probability of the release intervals (Figure 19 A\(_2\), grey lines through data points). Both the protein synthesis inhibited and control neurons have a fast and slow component, with the fast component accounting for
the majority of release event intervals in protein synthesis inhibited neurons ($t_{\text{fast}} = 143$ msec, 70%; $t_{\text{slow}} = 512$ msec) and the minority of release event intervals in control neurons ($t = 143$ msec, 27%; $t_{\text{slow}} = 512$ msec). Next, we delivered a tetanic stimulation (100 Hz, 4 sec) and measured the mEPSC frequency starting <4 sec after the tetanic stimulation. Interestingly, following tetanic stimulation, the frequency of spontaneous release is nearly identical for both control (6.1 ± 0.86 Hz) and protein synthesis inhibited (6.9 ± 1.2 Hz) neurons (Figure 19 B, B1, B2). This is also apparent in the cumulative probability histogram of the mEPSC intervals where the control and protein synthesis inhibited mEPSC curves partially overlap and no longer have a statistically significant difference (Figure 19 B2; p= 0.35, Kolmogorov-Smirnov test). Finally, although the fast and slow components of spontaneous release are still present following tetanic stimulation (Figure 19 B2), the fast component of spontaneous release increased to ~80 msec and the slow component increased to ~260 msec following tetanic stimulation for both control and inhibited neurons. Interestingly, following tetanic stimulation, the fast component accounted for approximately 70% of the frequency of spontaneous release events for both control and protein synthesis inhibited neurons. The finding that the cumulative probability of the release intervals nearly overlap following tetanic stimulation demonstrates that the effects of tetanic stimulation are not simply additive to the control and protein synthesis inhibited release intervals. This could indicate that protein synthesis inhibition and tetanic stimulation have similar mechanisms that produce faster mEPSC release rates, or that the mechanisms are not independent.
In contrast to the changes in spontaneous release, the amplitudes of the mEPSCs were unaffected by anisomycin treatment, tetanic stimulation or both combined (Figure 20 A, A\textsubscript{1} and B, B\textsubscript{1}). Furthermore, the mEPSC rise times (Figure 20 C), decay times (Figure 20 D), and area (Figure 20 E) were unchanged by tetanic stimulation, inhibiting protein synthesis, or both combined. Therefore the effects of inhibiting protein synthesis, and the effect of tetanic stimulation on mEPSC properties are specific to the rate of spontaneous release. Comparing the initial mEPSC intervals with the intervals following tetanic stimulation shows that tetanic stimulation increases the fast and slow rates of release, and percentage of rapid spontaneous release events (Figure 21 A). While previous activity affects the differences in the distribution of the release events between protein synthesis inhibited and control neurons, a tetanic stimulation greatly reduces these differences (Figure 21 B). Taken together, these results indicate that inhibiting protein synthesis has a purely presynaptic effect on spontaneous release. The increase in the rate of spontaneous release is similar in nature, but not in magnitude to how a tetanic stimulation affects spontaneous release.
Chapter 12

Discussion

The computational power of the brain is possible due to the remarkable connectivity between brain cells. Complex compartmentalization of neurons segregates different functions to individual sub neuronal structures, ultimately allowing neurons to convey, process and store information at distances far from the cellular somata. Local metabolic, structural and signaling requirements are quite different in different areas of the same neuron. This raises the question of how neurons are able to achieve such dynamic regulation of function with exquisite morphological complexity. This problem becomes even more significant when discussing the mammalian central nervous system (CNS), where axons can extend many centimeters. Furthermore, single neurons can have thousands of individual synaptic connections and these associations contribute to the processing power of the mammalian brain. However, it is the dynamic modification and regulation of synaptic connections that allow the brain to learn and adapt.

Translation is critical for the maintenance and regulation of the cellular proteome. Dendrites and axons have separate functions and occupy a rather large cytoplasmic volume that rivals the size of the cell body. Additionally, presynaptic nerve terminals can be extremely far distances from the cell body. Neurons have multiple methods to combat this complexity. Proteins can be translated in the cell body and cargo and adaptor proteins, with the assistance of chaperones, can be transported to certain subcellular addresses. Neurons also have the capability to
translate proteins in response to particular subcellular requirements. Allowing proteins to be spatially localized is the main advantage to controlling gene expression at the translational level. For efficient function, neurons evolved a mechanism to modify and regulate the local proteome in specific subcellular compartments. Local translation is the mechanism that allows the local proteome to be remodeled. The process of local translation is an extremely complex process and is carried out in multiple steps. Over the past few decades there has been a vast amount of work performed in elucidating local translation in dendrites and the postsynaptic neuron. Recently it has become accepted that local targeting of mRNA and local protein synthesis occurs in axons, growth cones, and presynaptic compartments. However, most presynaptic work has been performed in invertebrate and in vitro systems. Our work aimed to investigate if presynaptic protein synthesis occurs at an excitatory synapse located in the mammalian auditory brainstem, the calyx of Held.

It is worth noting that although previous work has shown local translation can occur in neurites, our work is showing the need for local presynaptic translation in a mature nerve terminal. In support of the ability for local translation to occur at mature presynaptic terminals, recent work has shown that ribosomes are present at an inhibitory synapse in the hippocampus and that local translation is required to initiate long-term depression (LTD).

Given the number of release sites and distance between the presynaptic terminal and the cell body, we hypothesized that local translation
may be necessary to maintain some protein components required for normal presynaptic activity. To test this we have shown a major component of ribosomes are present in the presynaptic terminal at the calyx of Held synapse (Figures 8, 9). This suggested that presynaptic translation could occur at this nerve terminal. However, this did not reveal if fully assembled and functional ribosomes are present at the calyx of Held nerve terminal. To address this we employed the SUnSET technique to show that ribosomes are not only present but also fully functional. This assay has previously been used to demonstrate local translation in dendritic, axonal and neuritic compartments in cell cultures. Due to the large size of the calyx of Held we were able to employ this technique in brain slice to show that presynaptic translation occurs (Figures 11, 12). Data obtained from the SUnSET assay indicate that fully functional ribosomes are present at the calyx of Held. Taken together these data provide compelling evidence that local protein synthesis occurs at the calyx of Held nerve terminal.

For the experiments performed in this study we chose to use P8 to P12 mice, because this allowed optimal imaging of the presynaptic compartment, and physiological properties have been extensively characterized during this time period. In addition this already functional nerve terminal is undergoing a morphological and physiological shift toward a mature synapse during this developmental period. Accordingly this requires the upregulation and downregulation of synaptic proteins that allow this presynaptic terminal to function at high frequency and maintain high fidelity synaptic transmission. This developmental transition continues over the next ~5 to 10 days, however it
becomes difficult to unambiguously identify the presynaptic compartment. Therefore, the P8 to P12 developmental window is optimal for the work done in this study.

The presence of functional presynaptic ribosomes suggests that protein synthesis may be necessary to maintain some aspects of synaptic transmission, at this synapse. In agreement with this we find that the initial frequency of spontaneous release is elevated in neurons where translation has been inhibited (Figures 13, 14). Interestingly, we find that there are fast and slow components of spontaneous release under control and conditions of translational inhibition. We found the fast component of initial spontaneous release to have an average interevent interval of ~170 msec (5-6 Hz), and the slow component to be ~750 msec (1.5 Hz). The important distinction is under control conditions the slow component was dominant accounting for ~65 % of the interval between events. In contrast, when protein synthesis was inhibited the faster component was dominant accounting for ~65 % of the interval between events. We speculate that this could be due to unregulated release under conditions of translational inhibition. It is possible a factor required to govern the spontaneous release of neurotransmitter, is not present or in limited amounts during period of protein synthesis inhibition. This suggests that ongoing protein synthesis is necessary for regulating the percentage of fast versus slow spontaneous release events.

The finding that inhibition of protein synthesis affects spontaneous release led us to the obvious question of effects on evoked release. Interestingly the
properties of single evoked events appear to be unaffected by inhibiting protein synthesis (Figure 15). However, the ratio of the amplitudes of paired stimulations revealed a reduced level of depression when protein synthesis was inhibited (Figures 16, 17). Furthermore, trains of stimulation (100Hz and 200Hz) also show a reduced level of synaptic depression in protein synthesis inhibited neurons compared to controls (Figure 18). Paired pulse ratios can reveal a lot about the initial probability (Pr) of release, and can be estimated based on the magnitude of the second response relative to the first. Mechanistically, the second response roughly depends on the intraterminal Ca\(^{2+}\) concentration \(^\text{118,149}\). Buffering of presynaptic Ca\(^{2+}\) after an influx event will determine the strength of the second response. If buffering is compromised, influx of Ca\(^{2+}\) during the second pulse will sum with residual thus increasing the amplitude of pulse 2. Our data show that more depression is seen in control conditions compared to anisomycin treatment. We speculate that this could be due to the inhibition of local translation of calcium buffers in neurons exposed to anisomycin. It is also possible that translation of calcium buffer interacting proteins that influence Ca\(^{2+}\) binding efficacy could also be an explanation. This indicates ongoing presynaptic protein synthesis also limits neurotransmitter release during stimulus trains.

Combined, the spontaneous and evoked neurotransmitter release properties suggest a protein(s) that are necessary to limit synaptic transmission are synthesized locally in the presynaptic terminal. Surprisingly the kinetics and amplitude of spontaneous and evoked responses appear to be unaffected during the time course (1-2 hours) of these experiments. This indicates that the effects we
observe are primarily due to translational inhibition in the presynaptic nerve terminal.

Our study was purposely limited to determine the early effects of inhibiting protein synthesis. In other experiments (data not shown) we have found some evidence for an increase in synaptic transmission failures and reduced maximum firing frequency after inhibiting protein synthesis. However, due to the variability in synaptic transmission over longer time courses we chose to perform blinded experiments on the initial effects of inhibiting protein synthesis on synaptic transmission. Since synaptic transmission properties change with activity, this suggests that inhibiting protein synthesis will have various effects on neurotransmission that change over time. In support of this we find that tetanic stimulation causes an increase in the time course and percentage of the fast component of spontaneous release events in control neurons (Figures 19, 20, 21). This results in the protein synthesis and control neurons having similar rates and percentages of fast and slow spontaneous release. We speculate that during a train of high frequency neuronal stimulation, calcium buffers and calcium sensors become saturated. This saturation would result in a phenotype that resembles conditions where levels or presence of certain calcium binding proteins were affected, similar to what we observe in neurons treated with translational inhibitor. This suggests that factors responsible for limiting neurotransmitter release are also affected following tetanic stimulation.
In conclusion, we have identified presynaptic ribosomes at the calyx of Held presynaptic terminal. Ribosomes are not only present under basal conditions but are also fully capable of translating peptides. Physiological maintenance of this extremely large presynaptic terminal could require local protein synthesis to regulate neurotransmitter release. Regulation of neurotransmitter release is important at any presynaptic nerve terminal located in the CNS, especially at a sensory synapse that needs to maintain well-timed synaptic responses at high firing frequencies. Transmission at a relay synapse must be fast, precise and reliable. The large caliber calyceal axon which projects to the contralateral MNTB, travels distances of ~2 mm in the mouse brain. Local translation allows for a rapid and dynamic response to modulate and control the local proteome. This bypasses the inefficient mechanism of slow axoplasmic transport for replenishment of some presynaptic protein cargo. Observations made in this dissertation could hold true for other presynaptic terminals located in the mammalian CNS.
Chapter 13

**Future Directions**

To further characterize local translation in the presynaptic compartment there are a few key experiments that could unravel a potential mechanism. The first step in understanding the phenomena of local translation at the calyx of Held is to identify the particular RNAs (mRNA, miRNA (micro RNA), shRNA (small hairpin RNA) that are targeted and localized to this nerve terminal. The large size of the calyx of Held makes this task possible, but still quite difficult. The use of a technique termed PatchSeq\textsuperscript{158} allows the use of a patch pipette to aspirate the contents of the nerve terminal. This can then be processed for RNA and high-throughput RNA sequencing (RNAseq) technology can be used to identify the particular species of RNA present in the nerve terminal. This can be done on basal (unstimulated) terminals and terminals stimulated electrically or chemically. The idea being that high frequency presynaptic stimulation could liberate heminodal or axonal RNAs that require a specific signal to fully localize to the terminal. This could yield a profile of nerve terminal RNAs whose function can then be probed with the use of siRNA (small interfering RNA). This is the most important next step to understanding the function and players involved, in local presynaptic protein synthesis.

The physiology data presented in this study revealed a decrease in short-term depression, determined with the use of paired stimuli. Short-term synaptic plasticity, at this synapse, has been extensively characterized\textsuperscript{118,149}. In brief, paired
pulse protocols deliver two stimuli at a fixed interpulse interval (5 msec or 10 msec, for our study). Taking the ratio between response 2 and response 1 will reveal the initial probability of release ($P_r$) for that neuron. For example, if the second response were larger than the first resulting ratio would be $>1$ or facilitated, this would be defined as the initial $P_r$ being low. Conversely, if the second response were smaller than the first resulting ratio would be $<1$ or depressed, this would be defined as the initial $P_r$ being high. Stimulation of a response causes an influx of calcium through voltage gated $\text{Ca}^{2+}$ channels. Calcium will then bind the calcium sensor for release thus completing synaptic vesicle fusion and release of neurotransmitter into the synaptic cleft. However, there is still residual calcium in the nerve terminal and the concentration depends on the amount and activity of intratermimal calcium buffers. Since the second pulse is delivered in rapid succession of the first (5 msec or 10 msec) calcium entering the terminal during pulse #2 can sum with the residual calcium from pulse #1 resulting in a bigger, second response. We speculate that decreased depression or facilitation seen in neurons treated with translational inhibitor is a result of defective or absent calcium binding proteins that are needed for proper and efficient buffering. Importantly sequencing data could provide evidence of this potential mechanism based on the identity of mRNA (messages for calcium binding proteins, calmodulin, etc.) However, there is a more straightforward way of testing our hypothesis for a potential mechanism. Administration of BAPTA-AM, a cell permeable calcium chelator, can be used to artificially buffer calcium during fiber stimulation in the presence of translational inhibition. If this reverses our observed phenotype, it
would suggest that calcium binding proteins (buffers) are not being locally produced when neurons are exposed to translation inhibition. In addition to the decrease seen in synaptic depression when neurons are exposed to translational inhibition we also observe an increase in the frequency of spontaneous release. We speculate that these data can also be explained by a defect in Ca\(^{2+}\) buffering. Furthermore, we believe that the normalization of the frequency of spontaneous release seen following tetanic stimulation is due to saturation in calcium buffering. Saturation in calcium buffering would cause the frequency in spontaneous firing in control neurons to phenocopy neurons treated with translational inhibitors. The idea is a saturation of calcium buffers would result in a similar phenotype to those same calcium buffers not being locally synthesized.

Finally, we need to provide more evidence that the effect we are seeing on synaptic transmission is exclusively presynaptic. The strength of the calyx of Held to study synaptic physiology lies in the accessibility to each side of the synapse with a patch pipette. This would allow us to inject a cell impermeant translational inhibitor (saporin, or cap binding protein analogs) to either the pre- or postsynaptic side. We can then apply the same stimulation paradigm as we did with bath application of inhibitor, and observe if we can produce the same phenotype. This would allow us to unambiguously determine which side(s) of the synapse is responsible for our observed phenotypes.
Figure 1. Anatomical connections of the auditory brainstem

Principal neurons located in the medial nucleus of the trapezoid body (MNTB) receive a single large excitatory input (the calyx of Held) that originates from globular bushy cells (GBCs) located in the anteroventral cochlear nucleus (AVCN). The large caliber calyceal axon creates a 1:1 axosomatic contact with neurons located in the contralateral MNTB. Principal neurons innervate many other brainstem nuclei and these neurons are inhibitory in nature. Principal cell projections are tonotopic in nature and represent low frequency and high frequency regions (indicated by the color gradient).

(Taken from Borst et al. 2012)
Figure 2. Stages of calyx development and maturation

(Left) Prior to calyx formation, principal neurons located in the MNTB are contacted at the soma and dendrites via divergent projections (~P2)

(Middle) An immature calyx of Held synapse (protocalyx) (~P5)

(Right) The mature calyx of Held synapse. It is important to note how morphology changes throughout calyx development. This calyx has a predominant spoon shaped morphology prior to hearing onset (~P10-11) and becomes highly fenestrated later in development (~P14). Along with morphological changes, the physiology of this synapse also changes with development. An example of this is the coupling of Ca\(^{2+}\) channels to release machinery. Another example is the shortening of the presynaptic action potential half width.

(Taken from Borst et al. 2012)
A. Most presynaptic nerve terminals in the brain are too small to be studied with conventional patch clamping techniques. Studying presynaptic activity in nerve terminals often requires inferences from postsynaptic responses. Most nerve terminals in the CNS are ~1 µm in diameter.

B. Differential interference contrast (DIC) microscopy depicting a single MNTB principal cell (red asterisk) engulfed by a giant spoon shaped presynaptic nerve terminal, the calyx of Held (white arrow). The calyx of Held nerve terminal can be ~10 µm in diameter.

C. The calyx of Held is accessible via a patch electrode (green) and can be distinguished from the postsynaptic MNTB using a tracer dye such as Lucifer Yellow, seen above. This synapse is amenable to dual pre- and postsynaptic recordings which allows the study of direct input output relationships. The postsynaptic neuron is filled with a rhodamine based dye and you can observe complete separation between pre- and postsynaptic compartments.

D. A diagram of the adult calyx of Held, containing its extremely large caliber axon (4-12µm in diameter). A typical bouton-type synapse is shown for comparison. It is important to note that in P8-P12 mice the calyceal axon traverses distances of up to ~2m.

(Adapted from von Gersdorff and Borst 2001)
Figure 4. Specialized compartmentalization of a neuron

Neuronal morphology depicting the neuronal cell body (gray), and neurites composed of dendrites (blue) and axons (red). The inset shows a synapse between the dendrite of one neuron and axon of another neuron. Efficient neuronal function is mediated by the collection and integration of signals received by dendrites, processing, and "decision-making" in the soma, and then transmission of information to the axons. Axons communicate to adjacent neurons at synapses where chemical transmitters released from the presynaptic terminal bind to receptors at the postsynaptic terminal of a dendrite. Synaptic communication is a highly regulated and dynamic process. Control of gene expression at the level of translation allows neurons to rapidly modulate the neuronal proteome. Localization of mRNA to certain neuronal substructures allows the neuron to respond rapidly and precisely through the use of on-site protein production.

(Taken from Rangaraju et al. 2017)
Figure 5. Experimental design for electrophysiology experiments performed in this study

Transverse brainstem slices are produced as described in Materials and Methods. Slices are perfused with normal aCSF (~2 mL/min) bubbled with carbogen. A bipolar fiber stimulator is then positioned at the midline where afferent calyceal axons cross. The image on the right shows a low power (20x) image of a fiber stimulator positioned on the brain slice (red arrow depicts location of the MNTB yield). The MNTB field is then scanned at ~29V (@1 Hz) until a single unit response is detected (image on the left is a typical unit recording seen from a connected neuron). A whole cell recording is then obtained in the voltage clamp configuration. The stimulus magnitude is raised 5V above threshold to ensure no failures in response. A typical calyx of Held synapse (P8-P12) should fire at 100 Hz with no failure. If there are no failures seen at 100 Hz we are then confident in our cell and slice health and proceed to switching out the solution lines to a blinded recording cylinder (DMSO or Anisomycin).
Figure 6. Stimulation Paradigm

The flow diagram seen above illustrates the stimulation paradigm that was used in this study. From initial break-in to completion of the stimulus protocol requires ~20 min. Stability of the recording is the rate limiting step in obtaining data that contain all physiological aspects measured from the protocol above. Each protocol is separated by ~2 min (2m). There are some instances where there is no time between protocols (0m) and this is because we wanted to test immediate effects of stimulation trains (100Hz or 200Hz). The stimulation paradigm was designed to obtain as much physiological data as possible from a single recording.
Figure 7. The calyx of Held displays distinct morphologies with progression through development.

Indirect immunohistochemistry (IHC) using an antibody directed against VGLUT1 specifically labels presynaptic calyx terminals and displays morphology. At postnatal day 8 (P8, top panels) the calyx is a large presynaptic terminal surrounding the postsynaptic principal neuron. By P16 (bottom panels), the dominant morphology is fenestrated with numerous swellings (scale bars: 50 µm or 10 µm) low magnification (left, 40x) and higher magnification (right, 100x) confocal images are shown for each age.

Figure 7
Figure 8. An integral ribosomal subunit is present at the calyx of Held nerve terminal

A. VGLUT1 antibody specifically labels presynaptic terminals from P11 mice brainstem slices. Immunolabeling of 5.8S rRNA shows the general location of ribosomes. Merged image shows overlay of VGLUT1 and 5.8S rRNA images. White arrows in all three panels depict several of the areas where a clear presynaptic 5.8S rRNA signal was present (confocal images, 40x).

B. Higher optical zoom (100x) of dotted box shown in Figure 6A. White arrows mark areas where 5.8S rRNA is present in a single presynaptic nerve terminal.

C. Line scan analysis depicts pixel intensity of VGLUT1 and 5.8S rRNA along a 15 µm line shown in Figure 6B. Merged line scans illustrate excellent overlap in relative signal intensity of VGLUT1 and 5.8S rRNA along the line.

D. Pearson’s correlation coefficients (r) used to quantify colocalization between the VGLUT1 and 5.8S rRNA signals, calculated for several presynaptic terminals (n=10). The r-value average of 0.78 ±0.02, demonstrates a high level of colocalization. The r-values for the matching postsynaptic cell body regions (n=10) were also calculated resulting in an average r-value of 0.11 ± 0.10. (p = ≤0.001)

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Figure 8
Figure 9. Nuclease treatment eliminates 5.8S rRNA signal indicating specificity of the antibody

A. Nuclease treatment prior to 5.8S rRNA antibody binding eliminates the ribosomal signal. Enhanced contrast further demonstrates the lack of ribosomal RNA signal following nuclease treatment (right panel). (40x, confocal image from P10 mouse)

B. Nuclease treatment greatly reduces the 5.8S rRNA signal compared to control conditions. (p = ≤0.001)
Figure 10. SUNSET (Puromycin structure and mechanism of action)

A. Comparison of the molecular structure of tyrosine, tyrosyl-tRNA, and puromycin. Puromycin mimics tyrosyl-tRNA and can be incorporated into a growing polypeptide chain. The major difference lies in the ester bond seen in tyrosyl-tRNA (blue arrow) which is hydrolyzable compared to the amide bond seen in puromycin (red arrow).

B. Mechanistically, puromycin incorporates into the growing polypeptide chain via the formation of a peptide bond. Once bound, the puromytyc can no longer elongate and is eventually released from the ribosome. Puromycin-bound polypeptides can be visualized using standard indirect immunofluorescence techniques. A primary antibody (1') directed against puromycin is used in combination with a fluorophore (red circle) conjugated secondary antibody (2').

(Modified from Trends in Biochemical Sciences, 28(3), TT Takahashi, RJ Austin and RW Roberts, "mRNA display: ligand discovery, interaction analysis and beyond", pp. 159-165)
Figure 11. Functional ribosomes are present in the calyx of Held presynaptic nerve terminal

A. VGLUT1 antibody was used to specifically label presynaptic terminals from P8 mice brain slices. SUnSET technique (see Figure 8) using an antibody against the tRNA analog puromycin specifically labels sites of active translation. Merged image, overlay of VGLUT1 and puromycin, show active ribosomes in pre- and postsynaptic compartments (confocal images, 40x).

B. Pearson's correlation coefficients (r) used to quantify colocalization between the VGLUT1 and puromycin signals, calculated for several presynaptic terminals (n=10). The r-value average of 0.74 ± 0.05, demonstrates a high level of colocalization. The r-values for the matching postsynaptic cell body regions (n=10) were also calculated resulting in an average r-value of 0.14 ± 0.03. (Error bars are s.e.m.) (p = ≤0.001)

C. Application of the translational inhibitor anisomycin (40 μM) for 1 hour prior to puromycin treatment eliminates puromycin labeling demonstrating the specificity of puromycin to binding actively translating ribosomes.

D. Anisomycin treatment greatly reduces the puromycin signal compared to control conditions. Inset shows residual ROI pixel intensity following anisomycin treatment. (p = ≤0.001)

E. (Top Panel) Higher optical zoom (63x) of the dotted box shown in Figure 9A. (Bottom Panel) Line scan analysis depicts pixel intensity of VGLUT1 and puromycin along a 10 μm line shown in Figure 9E Merge. Merged line scans illustrate excellent overlap in relative pixel intensity of VGLUT1 and puromycin along the line.
Figure 12. Additional example of functional presynaptic ribosomes at the calyx of Held nerve terminal

Additional example (top panel) of puromycylation (P8 mouse) which shows active ribosomes in both the pre- and postsynaptic compartments, demonstrated in line scans below each image. Merged overlay shows an excellent match in relative intensities of VGLUT1 and puromycin.

Figure 12
Figure 13. Initial Spontaneous release frequency is higher in neurons treated with protein synthesis inhibitor, indicating presynaptic effect

A. Initial spontaneous release in control (black) and neurons treated with the translational inhibitor anisomycin (red). Spontaneous events were recorded along a 30 sec continuous protocol. The entire 30 seconds is show in the representative traces seen above. (P9 mice)

B. (Top) Average mEPSC frequency are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red). Average frequency for control neurons (1.38 Hz ± 0.35) and anisomycin treated neurons (2.37 Hz ± 0.70). (Error bars are s.e.m. and differences seen are non-significant (ns)) (Bottom) Jittered X graph for the average values shown in top panel to illustrate the spread of the data. (n values are depicted in white (top panel) (# of animals/# of neurons)

C. Cumulative probability of spontaneous release event intervals between miniature excitatory postsynaptic currents (mEPSCs) in neurons treated with protein synthesis inhibitor (red) and control neurons (black). Value and amplitudes of double exponential fits (gray lines) are provided and show differences in the percent contributions of the fast and slow components.

Figure 13
Figure 14. Initial Spontaneous release amplitude and postsynaptic receptor kinetics are unaffected in neurons treated with the protein synthesis inhibitor anisomycin

A. Cumulative probability of mEPSC amplitudes in protein synthesis inhibited (red) and control (black) neurons. Inset depicts histogram of number of release events at different amplitudes.

B. Average mEPSC amplitude are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red). Average amplitude for control neurons (36.8 pA ± 2.5) and anisomycin treated neurons (38.7 pA ± 2). (Error bars are s.e.m.) (n values in bar graph apply to all panels in the figure (# of animals/ # of neurons))

C. Average mEPSC 10-90% rise time (left) and decay time (right) for control (black) and neurons treated with anisomycin (red). Average rise time for control neurons (0.76 msec ± 0.1) and anisomycin treated neurons (0.62 msec ± 0.04). Average decay time for control neurons (0.97 msec ± 0.13) and anisomycin treated neurons (0.73 msec ± 0.09). (Error bars are s.e.m.)

D. Average mEPSC area for control (black) and neurons treated with anisomycin (red). Average area for control neurons (40 pA*msec ± 4.7) and anisomycin treated neurons (32.4 pA*msec ± 2.6) (Error bars are s.e.m.)
**Figure 15.** Initial evoked response kinetics are similar in control and anisomycin treated neurons

A. Representative traces of evoked excitatory postsynaptic currents (EPSCs) for control (black) and neurons treated with anisomycin (red) from P10 mice.

B. Average 10-90% evoked EPSC rise time for control (black) and neurons treated with anisomycin (red). Average rise time for control neurons (0.73 msec ± 0.03) and neurons treated with anisomycin (0.67 msec ± 0.03). (Error bars are s.e.m.) (n values in bar graph apply to all panels in the figure (# of animals/# of neurons)).

C. Average 10-90% evoked EPSC decay time for control (black) and neurons treated with anisomycin (red). Average decay time for control neurons (0.86 msec ± 0.05) and neurons treated with anisomycin (0.75 msec ± 0.04). (Error bars are s.e.m.)

D. Average evoked EPSC amplitude for control (black) and neurons treated with anisomycin (red). Average amplitude for control neurons (5.73 nA ± 0.5) and neurons treated with anisomycin (5.13 nA ± 0.4). (Error bars are s.e.m.)

E. Average evoked EPSC area for control (black) and neurons treated with anisomycin (red). Average area for control neurons (5.99 nA*msec ± 0.62) and neurons treated with anisomycin (4.89 nA*msec ± 0.63). (Error bars are s.e.m.)

F. Average evoked EPSC latency for control (black) and neurons treated with anisomycin (red). Average latency for control neurons (1.99 msec ± 0.07) and neurons treated with anisomycin (1.85 msec ± 0.1). (Error bars are s.e.m.)
Figure 16. Reduced paired pulse depression with a 5 msec interpulse interval of evoked responses in neurons treated with the translational inhibitor anisomycin

A1. Representative initial paired pulse responses at a 5 msec interpulse interval (IPI) for control (black) and neurons treated with anisomycin (red). (Scale bar values apply to both traces). Traces shown are from P9 mice.

A2. Initial average paired pulse ratio (5 msec IPI) for control (black) and neurons treated with anisomycin (red). Control neurons have an average initial paired pulse ratio of (0.86 ± 0.08) compared to (1.05 ± 0.07) for neurons treated with the translational inhibitor anisomycin. Paired pulse ratios were calculated by dividing the second EPSC by the first (EPSC2/EPSC1). (Error bars are s.e.m.; differences seen are non significant (ns)) (n values in bar graph (# of animals/# of neurons))

A3. Paired pulse ratio (5 msec IPI) following a tetanic stimulation (100Hz for 4 sec) for control (black) and neurons treated with anisomycin (red). Control neurons have an average paired pulse ratio following tetanic stimulation of (0.66 ± 0.04) compared to (0.97 ± 0.06) for neurons treated with the translation inhibitor anisomycin. (Error bars are s.e.m.; p ≤ 0.001) (n values in bar graph (# of animals/# of neurons))

A4. Paired pulse ratio (5 msec IPI) ~ 5 min (recovery) following tetanic stimulation (100Hz for 4 sec) for control (black) and neurons treated with anisomycin (red). Control neurons have an average paired pulse ratio following recovery from tetanic stimulation of (0.73 ± 0.06) compared to (0.94 ± 0.05) for neurons treated with the translation inhibitor anisomycin. (Error bars are s.e.m.; p ≤ 0.01) (n values in bar graph (# of animals/# of neurons))

**Figure 16**
Figure 17. Reduced paired pulse depression with a 10 msec interpulse interval of evoked responses in neurons treated with the translational inhibitor anisomycin.

B1. Representative initial paired pulse responses at a 10 msec interpulse interval (IPI) for control (black) and neurons treated with anisomycin (red). (Scale bar values apply to both traces). Traces shows are from P10 mice.

B2. Initial average paired pulse ratio (10 msec IPI) for control (black) and neurons treated with anisomycin (red). Control neurons have an average initial paired pulse ratio of (0.62 ± 0.05) compared to (0.83 ± 0.06) for neurons treated with the translation inhibitor anisomycin. Paired pulse ratios were calculated by dividing the second EPSC by the first (EPSC2/EPSC1). (Error bars are s.e.m.; p ≤ 0.01) (n values in bar graph (# of animals/# of neurons))

B3. Paired pulse ratio (10 msec IPI) following a tetanic stimulation (100Hz for 4 sec) for control (black) and neurons treated with anisomycin (red). Control neurons have an average paired pulse ratio following tetanic stimulation of (0.59 ± 0.05) compared to (0.8 ± 0.04) for neurons treated with the translation inhibitor anisomycin. (Error bars are s.e.m.; p ≤ 0.01) (n values in bar graph (# of animals/# of neurons))

B4. Paired pulse ratio (10 msec IPI) ~ 5 min (recovery) following tetanic stimulation (100Hz for 4 sec) for control (black) and neurons treated with anisomycin (red). Control neurons have an average paired pulse ratio following recovery from tetanic stimulation of (0.61 ± 0.06) compared to (0.84 ± 0.06) for neurons treated with the translation inhibitor anisomycin. (Error bars are s.e.m.; p ≤ 0.01) (n values in bar graph (# of animals/# of neurons))
Figure 18. Anisomycin treated neurons exhibit a reduction in synaptic depression along a 200 Hz high frequency stimulus train

A. Representative traces of high frequency evoked EPSC trains at 200Hz for 400 msec for control (black) and neurons treated with anisomycin (red). Traces shown are from P9 mice.

B. Enlarged area of dotted box shown in Figure 18 A.

C. Average amplitude of first ten EPSC responses as a function of stimulus number during a 200 Hz 400 msec train, normalized to the amplitude of the first response in control (blue) and neurons treated with anisomycin (red) (p<0.01 for responses 2-8) and (p<0.05 for responses 9 and 10)
Figure 19. Spontaneous release following tetanus normalizes mEPSC frequency differences between control and protein synthesis inhibited neurons

A. Spontaneous release in control (black) and neurons treated with protein synthesis inhibitor anisomycin (red). Previous activity 400 msec trains at 100 and 200 Hz, and 100 Hz at 4 seconds produced a slight increase in the frequency of release in control neurons and treated neurons compared to initial mEPSC frequency (see Fig. 13A, B).

A1. (Left) Average mEPSC frequency are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red) before tetanic stimuli was applied. Average frequency for control neurons (2.5 Hz ± 0.5) and anisomycin treated neurons (3.6 Hz ± 0.9). (Error bars are s.e.m. and differences seen are non-significant (ns)) (Right) Jittered X graph for the average values shown in left panel to illustrate the spread of the data. (n values are depicted in white (top panel) (# of animals/ # of neurons)

A2. Cumulative probability of spontaneous release event intervals between miniature excitatory postsynaptic currents (mEPSCs) in neurons treated with protein synthesis inhibitor (red) and control neurons (black) prior to tetanic stimulation. Value and amplitudes of double exponential fits (gray lines) are provided and show differences in the percent contributions of the fast and slow components.

B. Spontaneous release in control (black) and neurons treated with protein synthesis inhibitor anisomycin (red) immediately following tetanic stimulation (100 Hz for 4 sec). (Scale bar is the same for both traces)

B1. (Left) Average mEPSC frequency are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red) immediately following tetanic stimulation. Average frequency for control neurons (6.1 Hz ± 0.8) and anisomycin treated neuron (6.9 Hz ± 1.2). (Error bars are s.e.m. and differences seen are non-significant (ns)) (Right) Jittered X graph for the average values shown in left panel to illustrate the spread of the data. (n values are depicted in white (top panel) (# of animals/ # of neurons)

B2. Cumulative probability of spontaneous release event intervals between miniature excitatory postsynaptic currents (mEPSCs) in neurons treated with protein synthesis inhibitor (red) and control neurons (black) immediately following tetanic stimulation. Value and amplitudes of double exponential fits (gray lines) are provided and show differences in the percent contributions of the fast and slow components.
Figure 20. Spontaneous release amplitude and postsynaptic receptor kinetics are unaffected in neurons treated with the protein synthesis inhibitor anisomycin before and after tetanic stimulation.

A. Average mEPSC amplitude are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red) prior to tetanic stimulation. Average amplitude for control neurons (39.8 pA ± 2.1) and anisomycin treated neurons (36.8 pA ± 2). (Error bars are s.e.m.) (n values in bar graph apply to all panels in the figure (# of animals/# of neurons))

A1. Cumulative probability of mEPSC amplitudes in protein synthesis inhibited (red) and control (black) neurons prior to tetanic stimulation. Inset depicts histogram of number of release events at different amplitudes.

B. Average mEPSC amplitude are shown for control (black) and neurons treated with the translational inhibitor anisomycin (red) immediately following tetanic stimulation. Average amplitude for control neurons (41.4 pA ± 1.9) and anisomycin treated neurons (38 pA ± 1.7). (Error bars are s.e.m.)

B1. Cumulative probability of mEPSC amplitudes in protein synthesis inhibited (red) and control (black) neurons immediately following tetanic stimulation. Inset depicts histogram of number of release events at different amplitudes.

C. Average mEPSC 10-90% rise time for control (black) and neurons treated with anisomycin (red) before (left) and immediately following tetanic stimulation (right). Average rise time for control (0.69 msec ± 0.07) and anisomycin (0.65 msec ± 0.04) treated neurons prior to tetanic stimulation. Average rise time for control (0.65 msec ± 0.03) and anisomycin (0.65 msec ± 0.05) treated neurons immediately following tetanic stimulation. (Error bars are s.e.m.)

D. Average mEPSC 10-90% decay time for control (black) and neurons treated with anisomycin (red) before (left) and immediately following tetanic stimulation (right). Average decay time for control (0.79 msec ± 0.09) and anisomycin (0.78 msec ± 0.08) treated neurons prior to tetanic stimulation. Average decay time for control (0.75 msec ± 0.07) and anisomycin (0.75 msec ± 0.07) treated neurons immediately following tetanic stimulation. (Error bars are s.e.m.)

E. Average mEPSC area for control (black) and neurons treated with anisomycin (red) before (left) and immediately following tetanic stimulation (right). Average area for control (36.9 pA*msec ± 4.6) and anisomycin (32.4 pA*msec ± 2.5) treated neurons prior to tetanic stimulation. Average area for control (35.7 pA*msec ± 3.1) and anisomycin (32.7 pA*msec ± 2.7) treated neurons immediately following tetanic stimulation. (Error bars are s.e.m.)
Figure 21. Summary of differences of spontaneous release events following treatment with the translational inhibitor anisomycin

A. Initial cumulative probability of release (dotted lines) compared to release immediately following tetanic stimulation in control (black) and neurons treated with anisomycin (red).

B. Difference in cumulative probability of release in anisomycin treated minus control neurons: initial difference (black); after brief activity (100 and 200 Hz stimuli for 400 msec, gray); before tetanic stimulation (blue); immediately following tetanic stimulation (green)
Bibliography


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