

**FUNCTIONAL IMPLICATIONS OF SNARE PROTEIN
INTERACTIONS WITH N-TYPE CALCIUM CHANNELS
IN THE *XENOPUS* NEUROMUSCULAR JUNCTION**

by

Ryan K. Keith

Bachelor of Science, Biological Sciences, University of Pittsburgh 2001

Submitted to the Graduate Faculty of

The Department of Neuroscience in partial fulfillment

Of the requirements for the degree of
Master of Science

University of Pittsburgh
2004

UNIVERSITY OF PITTSBURGH
FACULTY OF ARTS AND SCIENCES

This thesis was presented

by

Ryan K. Keith

It was defended on

February 24, 2004

and approved by

Dr. Yong Jian Liu

Dr. Stephen Meriney
(Committee Chairman)

Dr. Jon Johnson

Functional Implications of SNARE protein interactions with N-type Calcium Channels in *Xenopus* Neuromuscular Junction

Ryan K. Keith, BS

University of Pittsburgh, 2004

Classical Neurotransmitter (NT) release is dependent upon the influx of calcium (Ca^{2+}) through voltage gated Ca^{2+} channels. Once the local concentration of intracellular calcium increases to approximately 100 μM the calcium sensor detects the calcium ions and the process of vesicle fusion goes to completion. As such, transmitter release is very sensitive to alterations in the influx of Ca^{2+} into the presynaptic active zone. The objective of these experiments is to study how a known interaction between core complex proteins and presynaptic calcium channels might be important in the modulation of vesicle fusion.

Syntaxin 1A (stx1A) is part of the minimal protein machinery necessary for vesicle fusion. Stx1A has also been shown to interact with Ca^{2+} channels in vitro. The functional significance of this interaction however is unclear. Work in expression systems supports the idea that stx1A functionally interacts with the N-type Ca^{2+} channel causing it to become inactivated. It has also been proposed that the Ca^{2+} channel- syntaxin interaction functions to co-localize the release machinery in the vicinity of the Ca^{2+} channel. Essentially, this would put the trigger for release next to the source of Ca^{2+} influx.

To help discern whether this stx1A-N-type calcium channel interaction alters channel gating or simply serves to co-localize the two proteins, I employed a combination of molecular manipulations of syntaxin and the calcium channel. Electrophysiological recordings assaying NT release were then used to determine how these manipulations altered vesicle fusion. Data from two sets of experiments yielded the following information. 1) When the portion of the N-

type Ca^{2+} channel that binds with syntaxin 1A was injected into *Xenopus* embryos, the strength of NT release onto the postsynaptic cell appeared to decrease. This was suggested by increases in both paired pulse facilitation and tetanic potentiation after injection of the competitive peptide. 2) Recordings of paired pulse facilitation and tetanic potentiation with injection of a mutant form of stx1A, which couples with the calcium channel but which has been shown not to modulate the channel, suggest an increase in synaptic strength as assayed through a tendency for both paired pulse and tetanic potentiation to decrease following injections of the mutant. Taken together, these results suggest that the interaction between stx1A and N-type calcium channels influences the level of NT release. They do not however, definitively distinguish between the interaction strictly being structural or modulatory. Instead, the data suggests that both may be occurring in vivo and a balance between these modulatory influences determines that what may be physiologically important.

Acknowledgements:

I wish to thank my mentor Dr. Stephen Meriney for his guidance and support during my time as a graduate student in his laboratory. Without his help none of this would have been possible. I would also like to thank the rest of my committee; Dr. Yong-jian Liu and Dr. Jon Johnson for their helpful advice and support throughout this process. All the members of the Meriney lab have been amazingly encouraging and supportive and I would like to thank them as well. Lastly, I would like to thank my wife Elizabeth.

TABLE OF CONTENTS

Functional Implications of SNARE protein interactions with N-type Calcium Channels in <i>Xenopus</i> Neuromuscular Junction	iii
Acknowledgements:.....	v
1. Introduction.....	1
1.1. Synaptic Vesicle Fusion and Calcium Dependence of NT Release	1
1.2 Synaptic Vesicle Fusion, Functional Details and Components	2
1.3 Synaptic Protein Interactions with Ion Channels.....	3
2. Materials and Methods.....	9
3. Results:	15
3.1 Injection of synprint peptide into <i>Xenopus</i> embryos	15
3.2 Effect of Synprint Peptide Injection on Neurotransmitter Release.....	15
3.3 Effect of Mutant Syntaxin 1A mRNA on Neurotransmitter Release	18
4. Discussion	22
References.....	41

LIST OF FIGURES

Figure 1 29

Figure 2 30

Figure 3 31

Figure 4 32

Figure 5 33

Figure 6 34

Figure 7 35

Figure 8 36

Figure 9 37

Figure 10 38

Figure 11 39

Figure 12 40

1. Introduction

1.1. Synaptic Vesicle Fusion and Calcium Dependence of NT Release

The process of synaptic vesicle (SV) fusion and NT release is a fundamental building block of the nervous system. Without understanding how this process works, discussion of principles such as cognition and circuitry would be impossible. Thus it seems that alterations in the level of NT release should also be one of the logical places for plasticity in the nervous system. It is precisely this modulation that I wish to focus upon in this document.

A simplified view of the classical, chemical release of neurotransmitter from a presynaptic nerve terminal follows from the action potential invasion of a presynaptic nerve terminal. This causes a depolarization of the cell, which leads to activation of local presynaptic voltage gated ions channels (Katz et al. 1967). Calcium ions influx is predominantly responsible for triggering the NT release (Katz et al. 1967). Upon this flux intracellular levels of calcium increase, and once the local concentration of calcium is approximately $100\mu\text{m}$, vesicles at active zones (areas of the presynaptic terminal that are specialized for vesicle fusion) can be triggered to fuse. It is this fusion of the synaptic vesicle membrane and terminal plasma membrane that results in the liberation of NT, contained within the lumen of the synaptic vesicle, into the synaptic cleft. Intracellular calcium concentrations are not maintained for very long due to extensive homeostasis within neurons (Llinas et al. 1982). Excessive amounts of calcium are buffered by cellular organelles such as mitochondria, and endoplasmic reticulum (Bauer 2001, Naraghi et al. 1997). The complete process of vesicle fusion and NT release is very complex. It involves many different protein components and the role of each player isn't necessarily clear.

1.2 Synaptic Vesicle Fusion, Functional Details and Components

Essential vesicle release proteins minimally include vesicle associated membrane proteins termed (vSNAREs). The main protein of focus, which is minimally required for NT release, is synaptobrevin (VAMP) (Trimble et al. 1988). The plasma membrane also contributes crucial minimal proteins known as (tSNAREs). tSNAREs include the integral membrane protein syntaxin1A (stx1A), and a membrane associated protein, SNAP25 (Sollner et al. 1993, Oyler et al. 1988). SNAP25 is anchored to the terminal plasma membrane via palmitoylation of a cysteine rich linker region of the protein. Taken together these three proteins form the core complex. The core complex is a structural description of the molecular interaction between these three proteins. SNAP25 and stx1A interact with VAMP on the vesicle to form a stable ternary complex (Hayashi et al. 1994). Specifically, arginine residues from VAMP interact with glutamine residues on the syntaxin H3 domain and SNAP25 contributes two glutamine rich domains (Zhong et al. 1997). The interaction between these domains is highly hydrophobic and very stable. It is resistant to SDS treatment and once the complex is in this ternary configuration it is also resistant to cleavage by varying toxins, including the various strains of botulinum toxin (Pellegrini et al. 1995). This stable protein-protein interaction functions to bring the synaptic vesicle (SV) into close apposition with the plasma membrane of the presynaptic terminal. The synaptic vesicle also contains a protein called synaptotagmin. Synaptotagmin is an integral vesicle membrane protein, which has been shown to have two calcium binding domains, known as the C2A and C2B domains. It is thought that synaptotagmin could be the calcium sensor for vesicle fusion. When there is no increase in intracellular calcium, synaptotagmin is in a conformation allowing it to interact with the three members of the core complex. This complex functions to hold the vesicle in close apposition with the plasma membrane and to prevent

fusion. This point in the release process is termed priming. It is said that the SV is primed for release once the core complex proteins are interacting. Only the calcium trigger is needed for completion. Once intracellular calcium reaches appropriate concentrations ($\sim 100 \mu\text{M}$), it binds synaptotagmin, which changes conformation. It is this conformational change that causes synaptotagmin to cease interacting with the other core complex proteins and subsequently vesicle fusion goes to completion. It could be viewed that synaptotagmin may act as a clamp for vesicle fusion (Kee et al. 1996, Li et al. 1995). Once calcium flows into the terminal the clamp preventing release is removed and the process of fusion can occur. After vesicle fusion the vesicle membrane is retrieved from the plasma membrane via clathrin coated endocytosis or a non-clathrin mediated process known as “kiss and run” (Aravanis et al. 2003, Sollner et al. 1993). Figure 1 shows a representation of the release process including the proteins involved and each step of the SNARE hypothesis.

1.3 Synaptic Protein Interactions with Ion Channels

Aside from the interaction between SNAP25, syntaxin1A, and VAMP, there is evidence that these proteins interact with other proteins that have been shown to be important in vesicle trafficking, other vesicle processes, or endocytosis. For instance, it has been shown that SNAP25 can interact with synaptotagmin, N-type calcium channels, SNAP, and NSF (Sorensen et al. 2002, Zhang et al. 2002). VAMP can interact with AP2. Syntaxin1A binds to munc18, synaptotagmin, SNAP, N-type calcium channels, potassium channels, etc (Hodel et al. 1994, Yokoyama et al. 1997, Wu et al. 1994). Of particular interest are syntaxin's interactions with voltage gated calcium channels. At the frog neuromuscular junction the calcium channel

predominantly responsible for triggering NT release is the N-type calcium channel (CaV 2.2) (Rettig et al. 1997, Yazejian et al. 1997).

N-type calcium channels are multimeric proteins. They consist of four main subunits; alpha-1, alpha-2, beta, and delta. The alpha-1 subunit is the main pore forming subunit. The beta subunit is thought to be important for trafficking of calcium channels to the membrane as well as being important for expression at proper areas on the plasma membrane. The alpha-2 delta subunits are also thought to be important for trafficking and expression (Walker, Waard 1998).

Syntaxin has been shown specifically to interact with the alpha-1 subunit of the N-type calcium channel. The interaction is between the H3 domain of syntaxin and the “synprint site” on the calcium channel. The “synprint site” is a portion of the alpha 1 subunit, which is located on the II-III intracellular loop. This is an intracellular linker domain between the second and third domains of the alpha subunit (Mochida et al. 1996) (Figure 2B). Syntaxin’s structural coupling to the calcium channel functions to co-localize the source of calcium entry to the release machinery. This brings the probable calcium sensor, synaptotagmin; into close proximity to the source of high intracellular calcium that triggers transmitter release. The C2A domain of synaptotagmin has a calcium binding affinity in the 100 micro-molar range. (Figure 2A shows a representation of a synaptic vesicle including synaptotagmin and VAMP). Experiments have shown that when the interaction between N-type channels and syntaxin is altered by competitive synprint peptides, a shift in calcium dependence of release is observed, after synprint injection there is a 25% reduction in the transmitter release at normal extracellular calcium levels (Rettig et al 1997).

Figure 12 is our hypothesized working model of this interaction between stx1A and the N-type calcium channel through the synprint site. In the control state (Figure 12A) the N-type calcium channel can interact with the release machinery through stx1A. When a synaptic vesicle docks and primes with the plasma membrane, the stx1A-calcium channel interaction allows the trigger for fusion to be in a close vicinity to the source of calcium intracellularly. In an experimental state, which will be more fully discussed later, if an exogenous synprint peptide is present in the nerve terminal (Figure 12B) it has the ability to compete for binding to stx1A with endogenous N-type calcium channels. This perturbation of the native interaction in theory would cause a loss of co-localization between the release machinery and the channel. This loss of co-localization may result in the calcium sensor being less efficient in detecting increases in intracellular calcium due to the channel being farther away from the sensor than in control conditions.

There is evidence that syntaxin 1A can interact with the synprint portion of the calcium channel and that this interaction causes the ion channel to go into an inactivated conformation. This is hypothesized to occur when no synaptic vesicles are present at the active zone, because the release machinery in this state is capable of binding the calcium channel. This hypothesis follows that when there is a synaptic vesicle docked to the release machinery, syntaxin changes conformation and is no longer able to interact with the calcium channel. This allows the channel to go from an inactivated to an activated state, and thus the channel is more likely to open during AP depolarization and trigger NT release.

This hypothesis has been developed primarily as a result of the interpretation of expression system data. In these experiments, when a mutant form of syntaxin 1A is overexpressed, which structurally couples to the synprint portion of the Ca^{2+} channel, there is no

alteration of Ca^{2+} channel function. In contrast, when wild-type syntaxin 1A is co-expressed with the Ca^{2+} channel, slow inactivation of the channel is observed. The mutant is a two amino acid point mutation on syntaxin at amino acids 240 and 244. Amino acid 240 is alanine and the mutation changes it to a valine, whereas amino acid 244 is a valine and the mutant form switches it to an alanine (Bezprozvanny et al. 2000).

Figure 12A shows the control state. When no synaptic vesicle is present docked stx1A interacts with the N-type calcium channel to inactivate it. However when a synaptic vesicle docks syntaxin is hypothesized to change its conformation and this change results in a relief of inactivation on the N-type channel. This relief and subsequent influx of calcium ions results in efficient synaptic vesicle-plasma membrane fusion. In the case of over-expression the A240V,V244A stx1A mutant (Figure 12C) we see that stx1A structurally binds to the calcium channel but does not inactivate it. As a result we hypothesize that calcium influx through the channel may be even more efficient than in the control state. This would result in the calcium sensor detecting a larger increase in intracellular calcium and thus more NT release in this experimental state.

In summary, there is currently uncertainty about which potential effect of the syntaxin- Ca^{2+} channel interaction is occurring in vivo and is functionally relevant to the modulation of transmitter release. On one hand it would make sense that one would want to anchor the release machinery to the calcium channel but on the other, with the abundance of proteins present in the active zone you would think that it is unlikely that syntaxin would be the only protein anchoring these two necessary components of release together. Cytoskeletal structure is known to be present at the presynaptic terminal thus it is also unlikely that channels could simply float away from synaptic vesicle release sites although there may be subtle, yet significant rearrangements

when syntaxin is cleaved (Stanley et al. 2003). On the other hand, the idea of syntaxin being capable of communicating and modulating the calcium channel seems plausible. My thesis work seeks to provide data that will further our understanding of the functional significance of syntaxin- Ca^{2+} channel interactions at a neuromuscular synapse. To accomplish this, I have produced the double point mutant described above, and which has been used in heterologous expression systems to demonstrate altered calcium channel function. To complement the mutant data, I further explored the perturbation of the interaction between syntaxin and the N-type channel with the use of synprint peptides.

When the synprint portion of the calcium channel was injected into *Xenopus* embryos, the resultant tadpole tail neuromuscular junctions showed significantly increased paired pulse facilitation and tetanic potentiation. These recordings are measurements of short term plasticity in the presynaptic terminal. Paired pulse facilitation is thought to be caused by residual calcium left intracellularly when a second stimulus is presented a short time after the first. Data showing increased paired pulse facilitation and tetanic potentiation is often interpreted as indicative of a weaker synapse than in controls. Interestingly, stx1A mutant data revealed a trend in the opposite direction. When paired pulse and tetanic protocols were applied, after injection of mutant stx1A mRNA, a trend towards decreased short-term plasticity was observed. If these data become significant with further experiments (n=3 currently), the data would be interpreted to reflect stronger synaptic transmission. Furthermore, these data might support the idea that syntaxin functionally modulates the calcium channel. An examination of both synprint and syntaxin mutant data suggest that both co-localization and modulation may be occurring in vivo, and that ultimate effects on release depend on the balance of these influences. In order to more

fully understand the plasticity of neurotransmitter release both of these interesting findings will be explained in the context of how they may be altering physiology.

2. Materials and Methods

Plasmid subcloning: Rat Syntaxin1A in pRc/CMV was contributed by the laboratory of Dr. Ray Frizell, University of Pittsburgh. Custom sequencing primers were designed and sent to GIBCO BRL for production. Following confirmation of the rat stx1A sequence, the stx1A insert was removed from pRc/CMV with restriction enzymes HindIII and Xba1 (Promega). The DNA digest samples were loaded along with HindIII and X174 DNA markers into a LMP (low melting point) agarose gel and the gel was set to run at 60V for approximately 2 hours. The gel was then removed from the electrophoresis box and moved to a U.V. light box where the pGEM4z and stx1a bands were identified and excised from the LMP gel. The new pGEM4z plasmid and the stx1A were extracted using the QIAquick Gel Extraction Kit. The DNA slices were placed into a 1.7ml epindorf tube and spun in a microfuge for one minute. Three volumes of QX1 buffer were added to the slice and the sample was incubated at 50°C for 10 minutes. The dissolved sample was then placed in a spin column and collection tube and spun down (microfuge) repeatedly to purify. The columns were then spun for one minute to elute the DNA. Following the spin, 5 µl of 3M NaOAc, (pH 5.2) was added along with 137 µl of EtOH. The solution was then vortexed and allowed to precipitate at -20°C overnight. The following day the ligation reaction was performed to obtain the new stx1A/pGEM4z plasmid. The overnight precipitation was spun for 20 minutes to pellet the DNA. Following the spin the supernatant was removed and the pellet was dried. The pellet was then resuspended in 10 µl of TE (pH 7.4). The ligation reaction was then setup as follows: 13 µl dH₂O, 2 µl 10x rxn buffer, 2 µl of stx1A insert, 2 µl of pGEM4z, and 1µl of T4 ligase. The reaction was then stored at -20°C overnight.

Bacterial transformation: After ligation into the pGEM4z vector, the rat stx1A was transformed into DH5 E. coli competent cells for further workup and purification. This transformation was

done as follows. 100 µl of DH5 cells were pipetted into chilled Falcon 2059 double stop tubes. 5 µl of pGEM4z rat stx1A was then pipetted into the falcon double stop tube along with 6 µl of Ampicillin. The solution was then inoculated for one hour and spun down to pellet the cells. The pellet was resuspended in 100 µl of LB media. 15 µl of this suspension was pipetted onto an LB/Amp agar plate and placed in 37°C incubation for 12-16 hours. Following the incubation a single bacterial colony was removed from the plate using a sterile inoculating loop. The colony was placed in 0.5ml of ampicillin and 250 ml of LB media in a 500 ml flask. The suspension was then inoculated at 37°C for 12-16 hours and then purified.

Plasmid Purification: The pGEM4z rat stx1A plasmid was purified using Qiagen Maxi plasmid purification kit. The procedure consists of multiple treatments with varying pre-made buffers accompanied by high speed centrifuging to purify and elute the plasmid. The supernatant following elution was then poured off and the pellet was allowed to dry. The pellet was resuspended in 200 µl of T.E. at a pH of 7.4 and transferred to a 1.7 ml epindorf tube for storage at -20°C.

DNA/RNA production: Once the purified plasmid was obtained the stx1A DNA was linearized using XbaI and HindIII. The reaction was incubated for 2 hours at 37°C. 20 µl of sample DNA was added to 5 µl of ethidium bromide and loaded onto a 4% agarose gel. The gel was run at 60V for two hours. The identity of the DNA was verified using this DNA electrophoresis and visualized using a UV lamp. Once the identity of the stx1A was verified, production of RNA was accomplished using a T7 transcription kit. This process involves assembling linearized DNA with an RNA enzyme mix and reaction buffer and incubating the reaction at 37°C for 2 hours. The RNA is then recovered from the reaction using equal volumes of phenol chloroform and chloroform with the RNA. The aqueous layers of these equal parts are then precipitated in

isopropanol overnight and then pelleted via centrifugation. RNA identity was verified via RNA formaldehyde gel electrophoresis and UV visualization.

Syntaxin 1A mutant production: Custom primers were designed and sent away for production and sequencing verification (Gibco BRL). Using verified primers, mutant stx1A DNA was produced (Stratagene Quick Change Site Directed Mutagenesis, PCR). The mutation was two, two point mutations, A240V and V244A. Specifically, the manipulation was a switch 5'- 3' of GCT to GTT at amino acid 240 and a 5'- 3' GTG to GCT switch at amino acid 244. The alteration of these base pairs produced a unique restriction site for restriction enzyme Afl III (NE Biolabs). DNA electrophoresis revealed incorporation of this unique restriction site. To further verify the identity of the sample mutant DNA was sent away for sequencing (University of Pittsburgh BioMedical Research Support Facilities).

Synprint peptide purification: Synprint plasmid provided by Dr. William Catterall was subcloned into a pTrcHis vector and transformed into BL21 competent cells. The BL21 cells were then inoculated and induced to express protein. Synprint peptide was purified via Ni/agarose bead columns. Peptide identity and concentration was confirmed using SDS-PAGE gel and nitrocellulose blotting using an anti-T7 tag monoclonal antibody (Novagen) that recognizes a His tag incorporated into the pTrcHis vector. The His tag is a series of six histidine residue incorporated into the 3' terminal of the synprint insert. Ni/agarose beads specifically bind to His tagged proteins. The complex formed from this binding allows removal of impurities from the peptide; the purified synprint can then be eluted from the agarose columns. A secondary rabbit antibody was used against the T7 primary and enhanced chemoluminescence (ECL) was used to develop the western blot. The peptide was reconcentrated to 1mg/ml (Microcon Centrifugal Devices).

Peptide/mRNA injection: Synprint peptide was purified, concentrated, and injected into both blastomeres of 2-cell stage *Xenopus* embryos treated with 2% cysteine NFR (116mM NaCl, 10mM Hepes, 2mM KCl, 1mM MgCl₂, 1.8mM CaCl₂ pH 8.5) Injection was done while animals were bathed in 10% Normal Frog Ringer (NFR) +4% ficoll. A fluorescent dextran was co-injected along with the synprint peptide, 1 mg/ml of synprint was mixed 1:1 with the 200μM fluorescent dextran and injection success was determined by embryo fluorescence (Figure 4). Wild type or mutant syntaxin mRNA was diluted 1:750 with H₂O and mixed 1:1 with GFP mRNA and injected as a mixture with GFP fluorescence being used as a control for injection success. As a control for synprint injections, rat GST was concentrated to 1mg/ml with 150mM potassium acetate and injected into both blastomeres of two cell stage embryos. Tadpoles were left in injection dishes for 3 hours and then transferred to larger volume dishes containing 10% NFR for subsequent development. Following ~80 hours of development (stage 44-46, Nieuwkoop and Faber, 1967), the head and intestines were removed from the animal and the tadpole was subsequently skinned. Once the tail was fixed in the recording chamber, injection success was verified by observing the tail under fluorescent light. Figure 4A and 4C show synprint injected and uninjected animals as bright field images respectively. As can be seen in Figure 4B, animals which received the synprint / dextran co-injections, show a high level of fluorescence. Un-injected animals show no fluorescence, Figure 4D.

Tadpole Dissection: Stage 42-44 *Xenopus* tadpoles showing dextran or GFP fluorescence (to indicate injection success) were placed in 10% NFR w/ 0.2 mg/ml Tricain anesthetic. Embryos were then transferred to a dish containing 1X NFR for dissection. Stage 42-44 animals were used for recordings (3 days post injection). Tadpoles were skinned and head and intestines are

removed. They are then fixed with histoacryl glue to a recording dish for extracellular recordings.

Electrophysiological recordings: Patch electrode shaped pipettes (~4-6 μ m diameter tips) were fabricated and used as extracellular stimulating electrodes. N51A glass was used for these electrodes and pulled in a P-97 electrode puller (Sutter Instruments). Patch shaped recording electrodes were made of 1BBL glass (WPI). Recording electrode tip diameter was 3 μ m. 3 μ M Curare (Bachem) was used to partially block postsynaptic Ach receptors and prevent nerve-evoked contractions. Stimulating and recording electrodes were placed at opposite sides of the same chevron with recordings being done starting at the most rostral chevron and moving caudally with subsequent recordings. In each experimental animal, 30 Paired-pulse protocol recordings of E.P.P.s (Clampex 7 software) were done at each of the 10, 20, 40, 60, and 100msec inter-stimulus intervals. Each set of 30 EPP recordings at each inter-stimulus interval was averaged. In the same animals, 10-15 train stimuli recordings at 100Hz were recorded and averaged, following the paired pulse protocols. Train and paired pulse data were analyzed using ClampFit software. Data were plotted as amplitude of second EPP over amplitude of first EPP, for the paired pulse data. For 100Hz data, subsequent EPP amplitude was plotted over first EPP amplitude. Significance of data was determined via two population T-test (Origin, version 5). Figure 3 is a representation of microinjection, tadpole dissection, and electrode placement for extracellular recordings.

SDS Page Gel/ Western Blot: to assay for peptide presence *Xenopus* tadpoles were homogenized in 20 μ l per embryo, 100mM KCl, 0.1mM CaCl₂, 1mM MgCl₂, 10mM Hepes, 150mM NaCl, and 1% Triton X-100. Each embryo was triturated and centrifuged (14,000RPM, 10min @ 4°C). The supernatant of each embryo was loaded onto each lane of a 15% SDS page

precast gel, transferred to nitrocellulose overnight at 45mV. The nitrocellulose membrane was blocked in 5% nonfat milk with 1X PBST for 1 hour. The membrane was incubated in T7 antibody at a 1:5000 dilution in PBST for 1 hour and was then washed 3 times. Following the wash a secondary anti-mouse antibody at 1:3000 dilution in PBST was incubated with the membrane for 1 hour. Synprint bands were visualized by enhanced chemoluminescence (Pierce), with developing for exactly two minutes.

3. Results:

3.1 Injection of synprint peptide into *Xenopus* embryos

Molecular reagents were injected into two cell stage embryos and injection success was determined by fluorescence of experimental animals. To further confirm that the synprint peptide was still present, so that it has the opportunity to compete with the native syntaxin1A, N-type calcium channel interaction, I performed immunoblot analysis on both injected and control, dextran-injected embryos. Extracellular recordings were done on stage 44-46 tadpole tails. This corresponds to 3 days of development (Nieuwkoop and Faber, 1967). Exogenous synprint peptide has previously been shown to be present in *Xenopus* embryos 2 days post injection (Rettig et al. 1997). Immunoblot analysis showed that the exogenous peptide is still present at easily detectable levels at approximately 80 hours post-injection. Figure 5 (lanes 3-6) shows highly immunoreactive bands of 35kDA (Inoue et al. 1992), which corresponds, with the size of synprint peptide. His-tag antibody control antigen is shown in Figure 5 (lane 1). Also, of interest is that uninjected embryos show no immunoreactivity Figure 5 (lane 2).

Taken together, these pieces of evidence show that we are capable of injecting the synprint peptide into early stage *Xenopus* embryos, and the peptide is still present 80 hours later in vivo.

3.2 Effect of Synprint Peptide Injection on Neurotransmitter Release

As a way of assaying the effect of synprint injection on release I employed extracellular recordings of excitatory post-synaptic potentials (EPPs). By using this method of recording I was able to observe physiological effects of my experimental manipulations. There are a limited

number of causes for changes in postsynaptic potentials. These include altering postsynaptic receptors, changing quantal content, alteration of quantal packaging, or altering calcium dependence of release. Since there is no reason to believe that manipulating intracellular core complex proteins should alter postsynaptic receptors I ignored this possibility. There are few examples in which quantal packaging of neurotransmitter are the cause of changes in postsynaptic potentials. Since neither N-type calcium channels nor syntaxin have been shown to have a role in the filling of synaptic vesicles, it is unlikely that any change in EPPs would be due to differences in the amount of neurotransmitter within vesicles.

Two paradigms were used to assay exogenous synprint effect on neurotransmitter release. Because we expect alterations in presynaptic function, we employed paired pulse and tetanic stimulation paradigms as sensitive measures of these alterations. Paired pulse facilitation is a phenomenon that results from delivering 2 stimuli to the nerve terminal with a brief inter-stimulus interval. It is thought that calcium influx through calcium channels resulting from the first stimulation causes an increase in the effective intracellular calcium concentration so that upon the second stimulation, there is added Ca^{2+} leading to increased transmitter release. In extracellular postsynaptic recordings of EPPs, the second EPP amplitude is predicted to be larger than the first (if depression of release after the 1st stimulus is not significant). This is a measure of efficiency of fusion, release, and short-term plasticity within a neuron. Tetanic potentiation is a modification of the same principle. Again it is a measure of NT release efficiency and plasticity but instead of residual calcium being left from a single stimulation, tetanic potentiation is caused by accumulated residual calcium from repetitive stimulation that temporarily overcomes endogenous calcium buffers (Magelby 1987, Zucker 1989). It is known that the synprint site on the N-type calcium channel is the interaction site between the release machinery

and the calcium channel. Previous work has shown that injection of synprint peptide into *Xenopus* embryos decreased the level of neurotransmission recorded in cultured neuromuscular junctions by 25% (Rettig et. al 1997). Interestingly, in this earlier report no difference in paired pulse facilitation was observed. This lack of effects on facilitation is surprising. The group argues that the peptides are effectively uncoupling the release machinery from the area of localized calcium increase. This effectively would cause a reduction in the ability of the calcium sensor to detect local calcium concentration changes, which under normal circumstances would result in physiological levels of release. This would be predicted to result in a weaker synapse, and because weaker synapses have less depression, they would be expected to show more facilitation.

We repeated this experiment in developing tadpole NMJs in vivo. Figure 6A shows representative paired pulse, extracellular recordings of EPPs, at a 10msec inter-stimulus interval. The summary data are shown in Figure 6B. Paired pulse stimulation of *Xenopus* tadpole tail neuromuscular junctions revealed significant facilitation at an inter-stimulus interval of 10msec, (n=9, p= 0.0012) compared to control dextran, injected animals (n=15).

High frequency, tetanic stimulations were also used to determine efficiency of NT release as seen through the amount of NT being detected by postsynaptic receptors. Figure 7A shows representative extracellular EPP recordings done at a stimulus frequency of 100Hz. Each trace shown is an average of 10 sweeps. When the 100Hz data were compiled it became evident that there was also a significant increase in EPP amplitude with repetitive stimuli. Animals coinjected with dextran and synprint peptide had increased EPP amplitudes evoked by stimuli number 2-5(n=9, p<0.05) when compared to animals injected with dextran or GST peptide alone (n=5, n=4 respectively) (Figure 7B).

These pieces of data lead me to speculate that the neuromuscular junction injected with the synprint peptide has less efficient NT release than a junction, which is not injected with the peptide. This result could be due to any number of reasons and some likely possibilities will be discussed later.

To control for the possibility that the results shown from the injection of the synprint peptide are due to a nonspecific effect of a 35kDA protein, (the size of the synprint peptide), a GST peptide was co-injected with fluorescent dextran into 2-cell stage *Xenopus* embryos. After developing to stage 44-46, GST injected animals were dissected and extracellular paired pulse and tetanic stimulation protocols were performed as described above. The data for these injections can be seen in Figures 6 and 7. Representative traces using paired pulse and 100Hz protocols show very little difference when comparing GST injected animals to dextran injected animals (Figures 6A and 7A respectively). GST injection also showed little difference in tetanic potentiation (Figures 6B and 7B respectively). Thus, our results using the synprint peptide appear not to result from a nonspecific interaction between a 35kDA protein and the transmitter release components.

3.3 Effect of Mutant Syntaxin 1A mRNA on Neurotransmitter Release

Syntaxin 1A is necessary for normal neurotransmitter release. Cleavage of this protein with neurotoxins, such as Botulinum C toxin, results in an abolition of neurotransmission (O'Connor et. al 1997, Schiavo et. al 1995, Mochida et. al 1995). Syntaxin 1A interacts with proteins such as nSEC1, N (2.2) and L (1.2)-type calcium channels, cysteine string proteins, various G-proteins, as well as core complex proteins. (Kee et. al 1995, Wiser et. al 1996, Wu et. al 1999, Zamponi 2003). It is the interaction between the N-type calcium channel and syntaxin 1A that is the focus of this work. It has previously been shown that the H3 domain of syntaxin

1A interacts with a portion of the II-III intracellular loop on the 1B subunit of the calcium channels (Zhong et. al 1997, Atlas 2001). It is currently not clear whether this protein-protein interaction serves a structural co-localization role, or if syntaxin has a functional effect on the calcium channel.

There is some expression system evidence suggesting that syntaxin has the ability to modulate the kinetics of N-type calcium channels causing them to go into an inactivated state (Bezprozvanny et al. 1995, Wiser et al. 1996, Degtiar et al. 2000, Bergsman et al. 2000). Furthermore, there is evidence showing that a mutant form of syntaxin with point mutations at amino acid 240 (from alanine to valine) and amino acid 244 (from valine to alanine) causes syntaxin 1A to lose its functional modulation of the N-type channel (Bezprozvanny et al. 2000). I created this mutant for our studies in *Xenopus* tadpole tail NMJs in vivo. The identity of wildtype syntaxin1A was verified via DNA electrophoresis. Figure 8A shows wildtype stx1A in a pGEM4z vector linearized with restriction enzyme XbaI. The important detail is that the stx1A lane shows a band at approximately 35 kDA which is the appropriate size of syntaxin 1A (Bennett et al. 1992). Once syntaxin 1A was identified as being of adequate purity and identity, it was necessary to produce the desired mutations at amino acids 240 and 244. The mutagenesis at amino acid 240 had the benefit of incorporating an additional AflIII restriction enzyme site. This restriction site became useful in determining the identity and success of the mutation. Figure 8B is an electrophoresis DNA gel showing the presence of the AflIII site after mutagenesis. Note, in lanes three and four there is a single cleavage of stx1A when treated with AflIII. Lanes 5-12 show stx1A DNA after the mutagenesis. In each of these DNA samples there are two sites of AflIII cleavage, this corresponds with a successful mutation of the base pair, codons that make up amino acid 240.

As a further verification of the success of the two point mutations, the final DNA sample was sent to the University of Pittsburgh DNA sequencing facility for sequencing. The results of the sequencing can be seen in Figure 9A. The color peaks in the figure represent individual base pairs and the underlined letter on the top part of the figure identify the base pairs. The underlined GTT was mutated from base pairs GCT. This effectively switched amino acid 240 from an alanine to a valine. The second underlined codon shows a GCT. This GCT is a switch from the original GTG and this results in a switch of amino acid 244 from valine to alanine. As a final check of the identity of the mutant syntaxin 1A, after transcriptional production of *stx1A* mRNA, an electrophoresis RNA formaldehyde gel was run. Figure 9B shows the results of this gel. Lane 2 shows a band at the appropriate size for the syntaxin mutant. Since the identity of the syntaxin mutant was verified we were then able to proceed using the reagent to test for effects on transmitter release.

As has been stated, there is evidence that the mutant syntaxin 1A lacks the ability to promote an inactivated state of N-type calcium channels, but can structurally couple with the synprint site on the calcium channel (Bezprozvanny et al. 2000). Therefore, amino acids 240 and 244 are hypothesized to be within the region of syntaxin that is important for the modulation of calcium channel kinetics, but not binding. Mutant syntaxin mRNA was diluted 1:750 with dH₂O, mixed with GFP mRNA, and injected into 2-cell stage *Xenopus* embryos. Once the embryos reached stage 44-46 GFP fluorescence was used as a marker for injection success. Again, paired pulse and 100Hz stimulation paradigms were used as assays for synaptic strength. Figure 10A shows representative traces of extracellular, paired pulse EPP recordings. The trace on the left shows a control recording, injected with GFP mRNA alone. The right trace is an embryo injected with A240V, V244A mutant *stx1A* mRNA. As can be seen, in the representative data, it

appears that mutant injected animals show slightly less facilitation than control, GFP injected animals. Figure 10B represents summary data of paired pulse facilitation. Control animals, shown as red squares (n=15) appear to show more paired pulse facilitation than embryos, which were injected with the mutant syntaxin1A (n=3), however these data are not significant. If more experiments reveal a significant effect, these data can be interpreted to show that embryos that were injected with the A240V, V244A mutant may have stronger neuromuscular synapses than control tadpoles. 100Hz tetanic potentiation stimulation also showed a trend toward a decrease in EPP amplitudes following a first EPP (Figure 11A+B). Figure 11A shows representative 100Hz traces. The trace on the left is a control GFP mRNA injected embryo. The right trace shows an A240V, V244A / GFP mRNA injected embryo. Figure 11B shows compiled 100Hz tetanic potentiation data. Again, although these are not significant, if further experiments reveal significance, these data can be interpreted to suggest that an embryo injected with mutant syntaxin1A mRNA is stronger than a control GFP mRNA injected embryo.

4. Discussion

First, our data demonstrate that injection of molecular reagents into *Xenopus laevis* embryos at an early stage, in this case at 2-cell stage embryos, results in alterations in normal physiology and synaptic function at later developmental stages. Previous experiments suggested that injection of the synprint portion of the N-type calcium channel resulted in decreased neurotransmitter release in cultured synapses at, or below normal extracellular calcium concentrations (Rettig et al. 1997). These electrophysiological experiments were performed on ~48 hour old nerve-muscle co-cultures. 48 hours corresponds to a developmental stage of 32-34 (Nieuwkoop 1967). At stage 32-34 tail somites are poorly defined in vivo. This lack of definition makes finding neuromuscular junctions very difficult. Nerve terminals, which innervate tail somites, run along the somites and being unable to identify these terminal areas I find it exceedingly difficult to conduct successful extracellular EPP recordings (Seidman and Soreq 1997). In order to accomplish successful recordings of postsynaptic EPPs in vivo, slightly later developmental stages (44-46) were used. Stage 46 animals are approximately 75-80 hours old. Synprint peptides were previously shown to be present in high quantity in stage 32-34 tadpoles (Rettig et al. 1997). To verify that injected peptide was present at 80 hours, we performed Western blots on injected embryos at stage 44-46. Figure 5 shows that the injected synprint peptide is present at relatively high quantities at this developmental stage. In the case of syntaxin 1A, the presence of a coinjected GFP mRNA at the time of recording was used as an indicator of the syntaxin being present as well. Since there is no evidence that GFP has a significantly shorter half-life than syntaxin we believe that the manipulated syntaxin is also present at stage 44-46.

Thus, we have demonstrated that we are able to successfully manipulate embryos and that tadpoles show the presence of the manipulated reagents at a stage in which extracellular recordings can successfully be done. With this successful protocol established, we interpret differences in release to synprint or syntaxin altering normal synaptic function.

In order to fully interpret the electrophysiological data we must first review the hypothetical framework for the process of release. In a simplified model, the synaptic vesicle contributes VAMP and synaptotagmin, interact with plasma membrane proteins SNAP25 and syntaxin1A. Without any stimulus present these proteins interact loosely to bring the synaptic vesicle membrane into close vicinity with the plasma membrane (Chen et al. 2001). When the presynaptic terminal is depolarized, this causes voltage gated calcium channels to move into an activated state. Calcium flows into the presynaptic terminal, increasing intracellular calcium concentration to approximately 100 μM in the area immediately around the mouth of the calcium channel. There is evidence suggesting synaptotagmin's interaction prevents vesicle and plasma membrane fusion from occurring. Synaptotagmin also has C2 domains which have been shown to bind calcium ions. It is hypothesized that once calcium binds to the C2 domains, synaptotagmin changes conformation which prevents it from interacting with SNAP-25, VAMP, and syntaxin 1A any longer (Fernandez-Chacon et al. 2001, Kee et al. 1995, Li et al. 1996).

Syntaxin 1A interacts with the N-type calcium channel through the synprint site. This has a clear significance in that it localizes the release machinery and possible calcium sensor with the calcium source. In vitro binding studies have demonstrated the interaction (Bennett et al. 1992), and our extracellular data in vivo provide functional evidence of such an interaction in natural nerve terminals. When the synprint portion of the N-type calcium channel was injected

into the *Xenopus* embryos there was a significant increase in paired pulse facilitation (Figure 5). This was accompanied by an increase in tetanic potentiation when the manipulated tadpoles were stimulated at 100Hz. Since these recording paradigms are a measurement of short term plasticity resulting from residual intracellular calcium, we hypothesize that the synprint peptide is weakening the synapse due to less calcium at the calcium sensor for release. This could be due to either a decrease in calcium influx as a result of Ca^{2+} channel modulation or a drifting of calcium channels away from the sensor for release. The interaction between the H3 domain on syntaxin and the II-III intracellular loop on the calcium channel is known to be important to colocalize these proteins, but it is also possible that the interaction alters Ca^{2+} channel function. Of course, both co-localization and functional modulation could be occurring in vivo. With these recordings we have no direct insight into the peptides effect on N-type calcium channel inactivation. Since the peptide mimics a portion of the channel, it is unlikely that the injected peptide would be binding to and inactivate the channel, causing the observed results. The observed increase in paired pulse facilitation can however be explained if by injecting the peptide we interfere with the native interaction between syntaxin1A and N-type channels. With this interaction disrupted it is possible that the co-localization between the two elements may be lost. Experiments done in rat superior cervical ganglion neurons also show increased paired pulse facilitation along with decreased synchronous neurotransmitter release, and increased asynchronous release (Mochida et al 1996). Further experiments have shown that with injection of exogenous synprint peptide into 2-cell stage *Xenopus* embryos, there is a 25% decrease in synchronous neurotransmitter release in cultured synapses at 1.8mM extracellular calcium (Rettig et al. 1997). Rettig's group also modeled the effect of channels drifting slightly away from release machinery. The results of their model fit with the physiological data suggesting to

them that co-localization changes underlie the release results. More recent experiments using freeze fracture and electron microscopy have provided evidence that when botulinum toxin C1 is used to cleave the cytoplasmic portion of syntaxin that membrane particles may change localization slightly, between 3-5nm (Stanley et al. 2003). These pieces of evidence along with our data suggest that it is likely that the interaction between syntaxin and N-type calcium channels includes a structural co-localization.

Figure 12A is the complete, working, conceptual model showing what is hypothesized to be occurring in normal terminals. Without a synaptic vesicle present, there is no interaction between syntaxin, VAMP, and SNAP-25. In this configuration, syntaxin is able to interact with the N-type calcium channel. When a synaptic vesicle associates with the release machinery, syntaxin, SNAP-25, and VAMP interact to form the stable core complex. This interaction changes the conformation of syntaxin 1A in such a way that it cannot interact with the calcium channel. Calcium flows in through the calcium channels and triggers fusion of the synaptic vesicle to the plasma membrane. In the case of synprint injection (Figure 12B), the synprint peptide interferes with the exogenous interaction between the calcium channel and the release machinery. This interference possibly causes the N-type calcium channel to drift away from the release machinery. In light of the evidence that the synprint site interacts with other proteins such as SNAP-25, CSP, etc. the peptide could also be interfering with these interactions. This interference would make the model more complex but would not alter the general idea that synprint peptides effectively sever the interaction between the channel and all the co-localization machinery. When a vesicle docks at the release site under this condition, the calcium sensor has a reduced ability to detect calcium influx due to a greater distance between Ca^{2+} channels and

Ca²⁺ sensors. Thus, even if more Ca²⁺ channels are opening, the increased distance dominates the function of the synapse.

The other hypothesis states that along with the structural co-localization, there is also a functional aspect to the interaction between syntaxin and N-type calcium channels. Experiments done in *Xenopus* oocytes support the idea that syntaxin1A modulates N-type calcium channels, causing them to go into a slow inactivated state (Degtiar et al. 2000, Bezprozvanny et al. 1995). Further experiments provide evidence that there are two separate regions of syntaxin 1A that interact through the synprint site and by mutating specific amino acids within the H3 domain of syntaxin you can affect functional modulation of the N-type calcium channel by syntaxin without altering its ability to bind to the channel. The first region is the cytoplasmic portion of the H3 domain on syntaxin. This region is thought to be important in the structural binding of syntaxin to the N-type channel. The second area of syntaxin that appears to be crucial to a functional interaction is a region of amino acids, approximately 187-250, which by mutation analysis has been shown to change the amount of modulatory action syntaxin can have on the channel. A specific mutant, known as s1AYK6, has amino acid point mutations, A240V, V244A. This mutant bound to N-type channels but showed no ability to modulate the channel (Bezprozvanny et al. 2000). These previous mutation experiments were performed in *Xenopus* oocytes. In order to more fully explore if syntaxin 1A has a functional interaction with N-type channels besides the structural one described previously, we performed experiments in a natural synapse; *Xenopus* neuromuscular junctions in vivo. Our results provide a hint that such a functional interaction is also occurring in vivo. In vivo paired pulse and tetanic potentiation data with an injection of A240V, V244A mRNA showed a trend towards a decrease in EPP amplitude, under paired pulse and tetanic potentiation stimulation paradigms. If further data collection results in a significant

effect, these data can be interpreted as a strengthening of the synapse. At this point however, we have to conclude that there are no significant effects. Figure 12C shows the conceptual model for the interpretation of data following injection of mutant mRNA. In the case of the mutant, without a synaptic vesicle present at the release site, syntaxin physically interacts with the N-type calcium channel but there is no modulation. The calcium channel is therefore available to open with action potential depolarization even in the absence of vesicle docking. Thus, there may be a larger quantity of calcium influx than at a control site, in which syntaxin can move a fraction of the Ca^{2+} channels into an inactive state. This model would fit nicely with our results if we can demonstrate significance with further data collection.

In summary, the data suggest that the interaction between syntaxin 1A and N-type calcium channels through the synprint site may be more complex than previously thought. The interaction likely functions to co-localize the release machinery near the source of calcium influx, however, it is unlikely that this interaction is the only thing holding these two components of neurotransmitter release together, since there is a wealth of cytoskeletal elements that anchor other terminal proteins and could co-localize these proteins. It is possible however, that disruption of the syntaxin- synprint interaction causes a slight shift in localization and that this slight movement could result in an alteration in the calcium sensor's ability to detect intracellular calcium. Without structural data one can only speculate that this is occurring. It is encouraging however that perturbation of the interaction between core complex proteins and the calcium channel results in data that support our hypothetical model.

Interestingly, our data lead us to hypothesize that the interaction between these two proteins may have a modulatory effect. It appears that native syntaxin Ca^{2+} channel interactions may cause a decrease in NT release. Our results suggest that syntaxin modulates N-type calcium

channels, and that this modulation can be abolished through manipulation of regions of syntaxin that are responsible for it. At this point there is no significant difference between control and stx1A mutant animals but there does appear to be a definite trend towards a decrease in release in the presence of A240V,V244A.

Thus, it appears that the syntaxin 1A synprint interaction functions as a modulator for release and that the level of neurotransmitter release may be determined by a balance of influences. If calcium channels and release machinery proteins are tightly coupled, the calcium sensor is in a better position to identify increases in intracellular calcium. With this tight localization also comes an inactivation of the channel by syntaxin. The sensor is closer to the increase in calcium but less calcium is coming in due to functional modulation of the channel by syntaxin.

This intriguing complexity makes sense in a system in which multiple sites of modulation may be useful. Both the structural and functional aspects of the interaction between the N-type calcium channel and stx1A provide useful insight into possible sites for modulation. One must use caution when interpreting the results. We have examined an interaction between rat syntaxin 1A in a *Xenopus* system. *Xenopus* syntaxin 1A is not currently cloned, but there is a high syntaxin 1A homology between species (~90%). The *Xenopus* genome project may permit one to repeat these experiments when *Xenopus* syntaxin1A is cloned. It is also possible that there are unknown interactions between release machinery proteins and calcium channels though unknown binding partners. This would alter our working model but would not change the significance of modulation of the N-type calcium channel by syntaxin.

Figures:

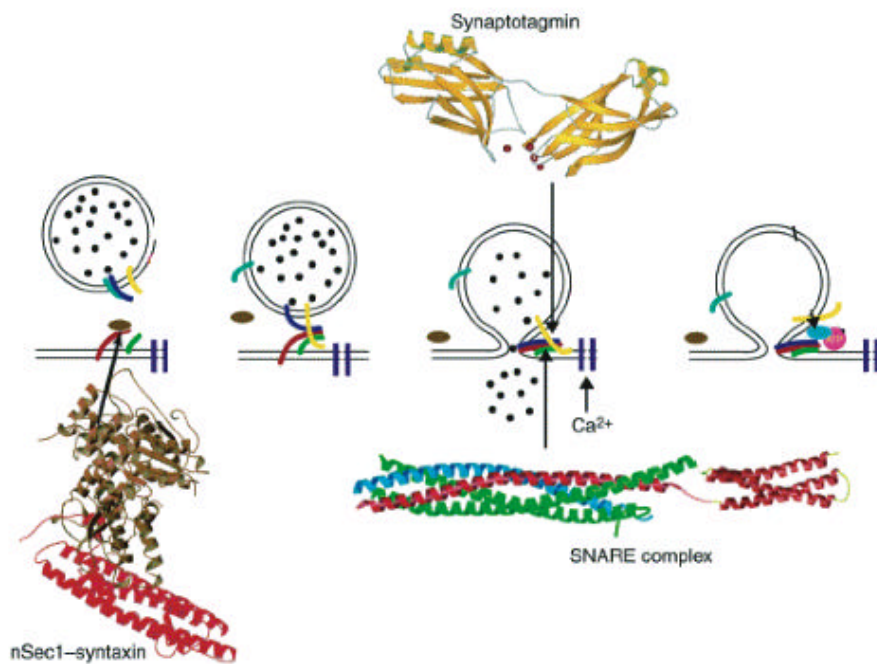
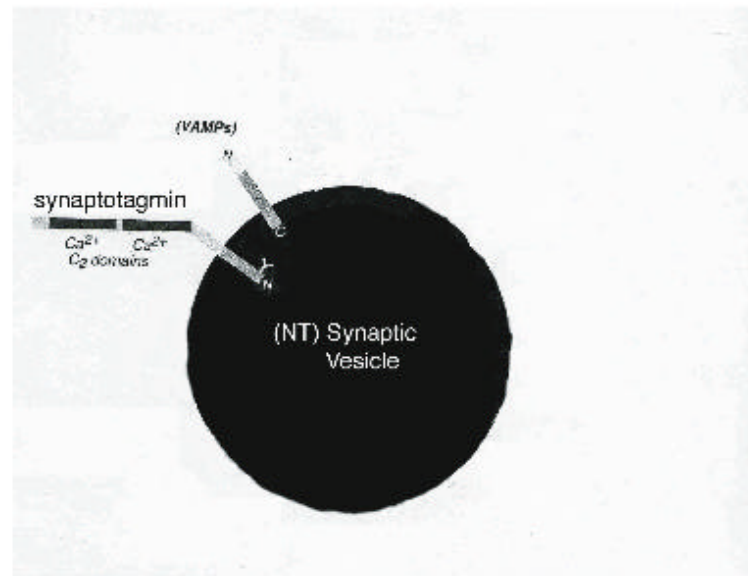


Figure 1: Representation of the SNARE hypothesis: pertinent release proteins are shown above and below a mechanistic model for release. In the first mechanistic picture the synaptic vesicle is trafficked to the terminal plasma membrane (docking). In the second picture vSNAREs, VAMP and synaptotagmin interact with tSNAREs, syntaxin1A and SNAP-25 (priming). In the third mechanistic picture calcium binds to the calcium sensor for release and fusion goes to completion. Adapted from Brunger 2000.

Figure 1

A**B**

N-type Calcium Channel Alpha Subunit

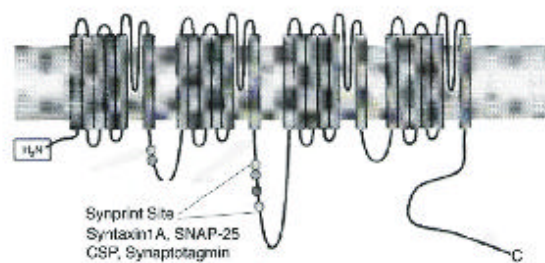


Figure 2: (A) representation of a synaptic vesicle and its associated proteins. Note synaptotagmin and its C2 calcium binding domains, also note Synaptobrevin (VAMP) Adapted from Sudhof et al. 2001 (B) Representation of the N-type calcium channel; note the Synaptic protein interaction site (Synprint). Adapted from Zamponi 2001.

Figure 2

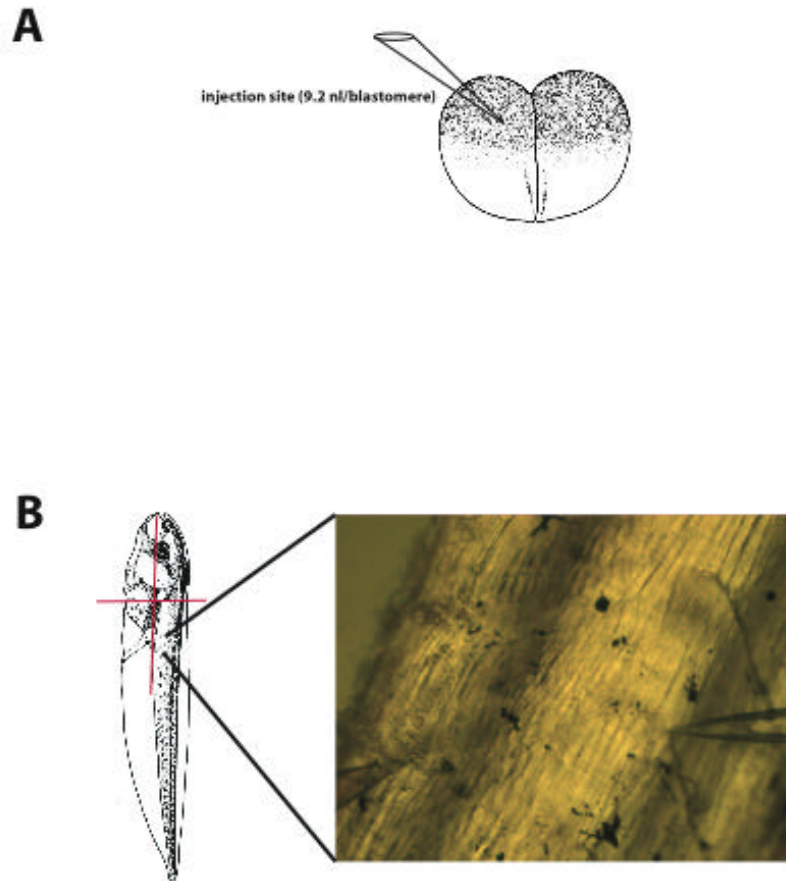


Figure 3: Methods: Tadpole manipulation: (A) Diagram representing microinjection of mRNA or peptide into 2-cell stage *Xenopus* embryos. Each blastomere of the 2-cell stage embryo received an injection volume of 9.2nl of varying solutions. (B) Diagram represents method of dissection, shown in red lines and (inset) placement of extra-cellular electrodes for EPP recordings.

Figure 3

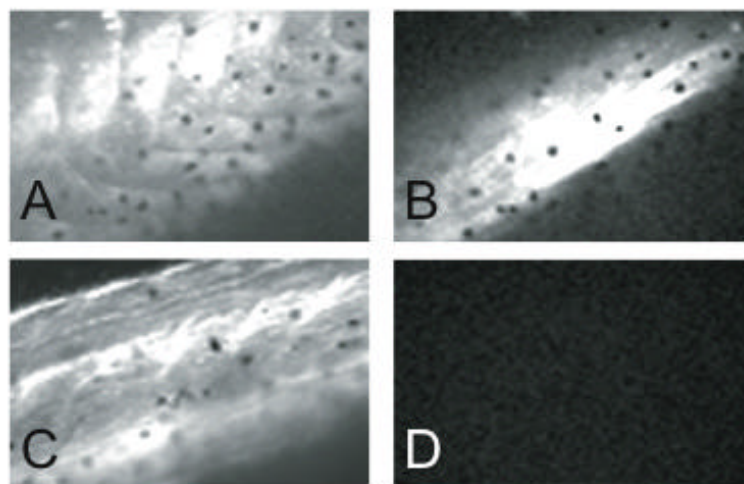


Figure 4: Injected embryos identified by the presence of fluorescent dextran. (A) Bright field image of an injected embryo. (B) Fluorescent image of injected embryo. (C) Bright field image of control (uninjected) embryo. (D) Fluorescent image of control (uninjected) embryo.

Figure 4

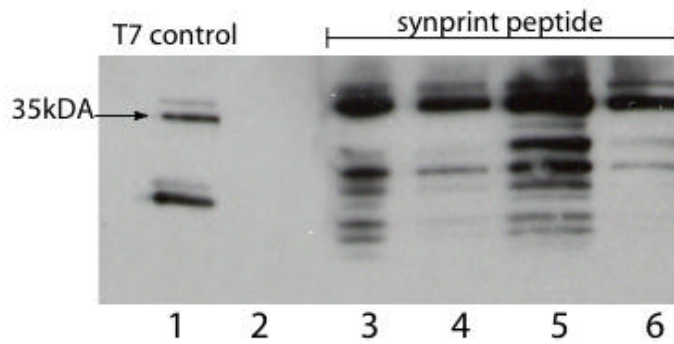


Figure 5: Western Blot of homogenized stage 44-46 (~3 days, 15 hours) *Xenopus laevis* embryos. Detection done using aT7 his-tag antibody (Novagen). Note the bands in lanes 3-6 which correspond with the T7 control (lane 1). The synprint bands are approximately at 35kDA which corresponds with the size of the synprint portion of the N-type calcium channel. Note, absence of bands in lane 2, which corresponds with an uninjected animal.

Figure 5

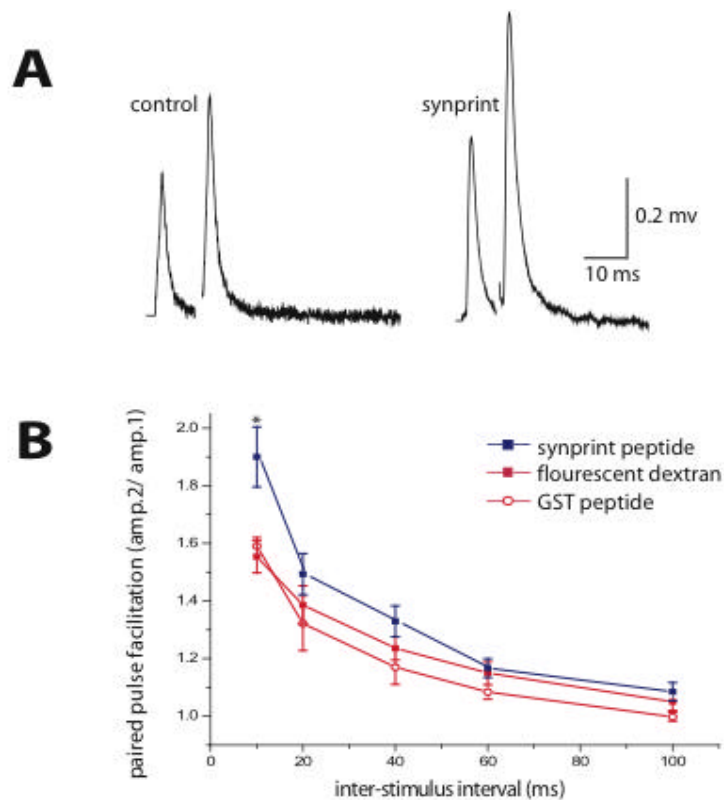


Figure 6: Injection of synprint peptide into 2-cell stage *Xenopus laevis* results in increased paired pulse facilitation of EPPs. (A) Representative EPPs; paired pulse facilitation recordings with a 10ms interstimulus interval. Left trace: control animal; Right trace: synprint injected animal. (B) Compiled data; synprint injection increased paired-pulse facilitation. Control PPF represented by red squares (n=15), and synprint injected animals represented by blue squares (n=9; * significance determined by t-test, $p=0.0012$). As a control for nonspecific protein effects, rat GST was injected along with dextran. GST animals show no significant difference compared to dextran injected animals (n=4).

Figure 6

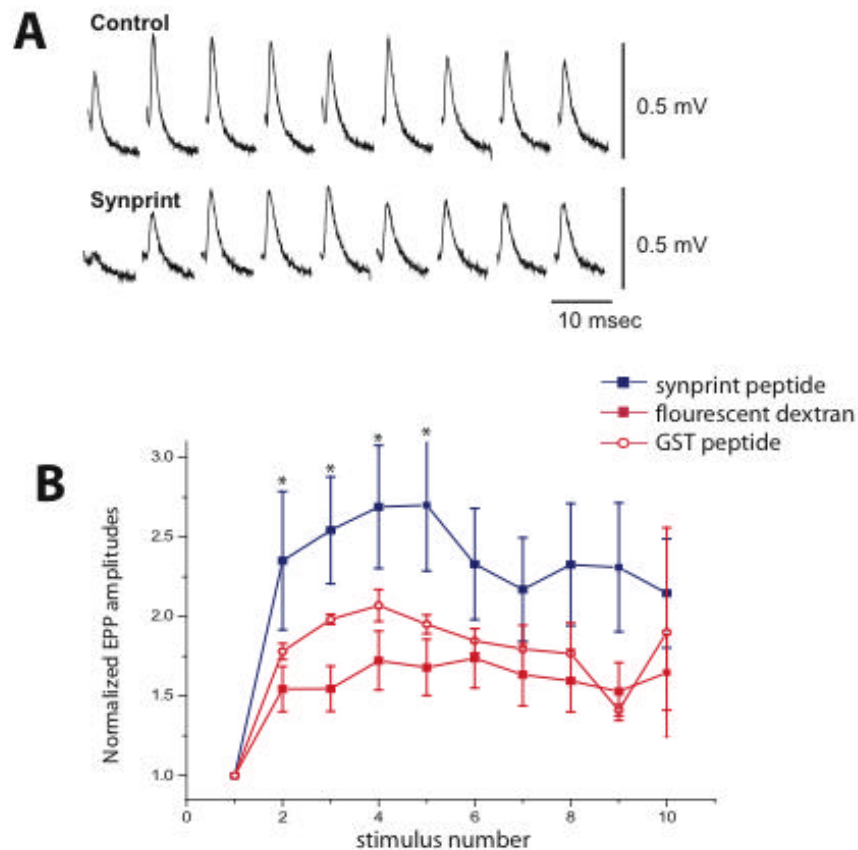


Figure 7: Injection of synprint peptide into 2-cell stage *Xenopus laevis* results in increased tetanic potentiation of EPPs. (A) Representative EPPs; recorded during 100Hz stimulation. top trace: control animal, average of 10 sweeps; bottom trace: synprint injected animal, average of 10 sweeps. (B) Compiled data; synprint injection increased tetanic potentiation. Normalized EPP size during 100Hz stimulation. Control fluorescent dextran animals represented by red squares (n=5); synprint injected animals represented by blue squares (n=9). To control for nonspecific protein interactions a GST peptide was also used as a control, represented by open red circles (n=4) *significance between synprint and control determined by t-test, $p < 0.05$).

Figure 7

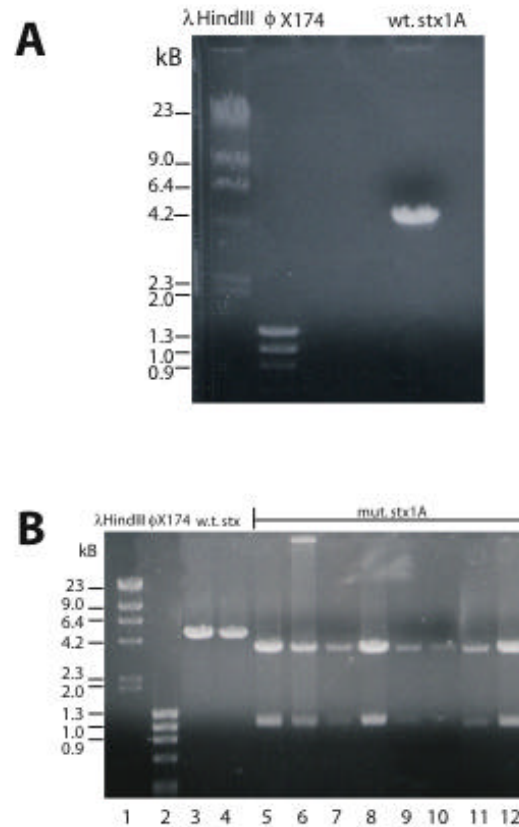


Figure 8: Identity of syntaxin 1A. (A) DNA gel electrophoresis showing syntaxin 1A band around 35kDa which appropriately corresponds with the known size of syntaxin 1A. This was accomplished using XbaI restriction enzyme following subcloning into a pGEM4z vector. (B) DNA gel electrophoresis of syntaxin 1A following site directed mutagenesis. Lanes 3 and 4 show wild type stx1A linearized with AflIII. Mutagenesis, (A240V,V244A) incorporated a second AflIII restriction site. Seen in lanes 5-12.

Figure 8

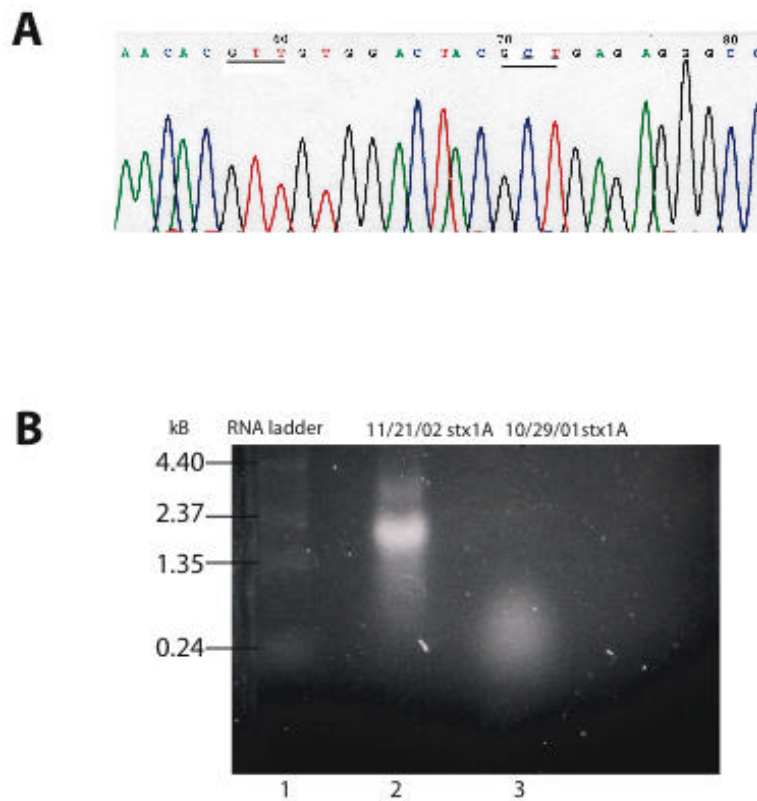


Figure 9: Identification of manipulated syntaxin 1A. (A) Sequencing showing the desired base pair mutations, The first mutation was a GCT to GTT switch shown underlined around base pair 60. This mutation resulted in an amino acid switch A240V. The second was a switch from GTG to GCT, shown underlined around base pair 70. This mutation resulted in an amino switch V244A. (B) An RNA electrophoresis gel showing the newly made mutant, A240V, V244A stx1A mRNA, lane 2. Lane 3 is a degradation product of stx1A mRNA.

Figure 9

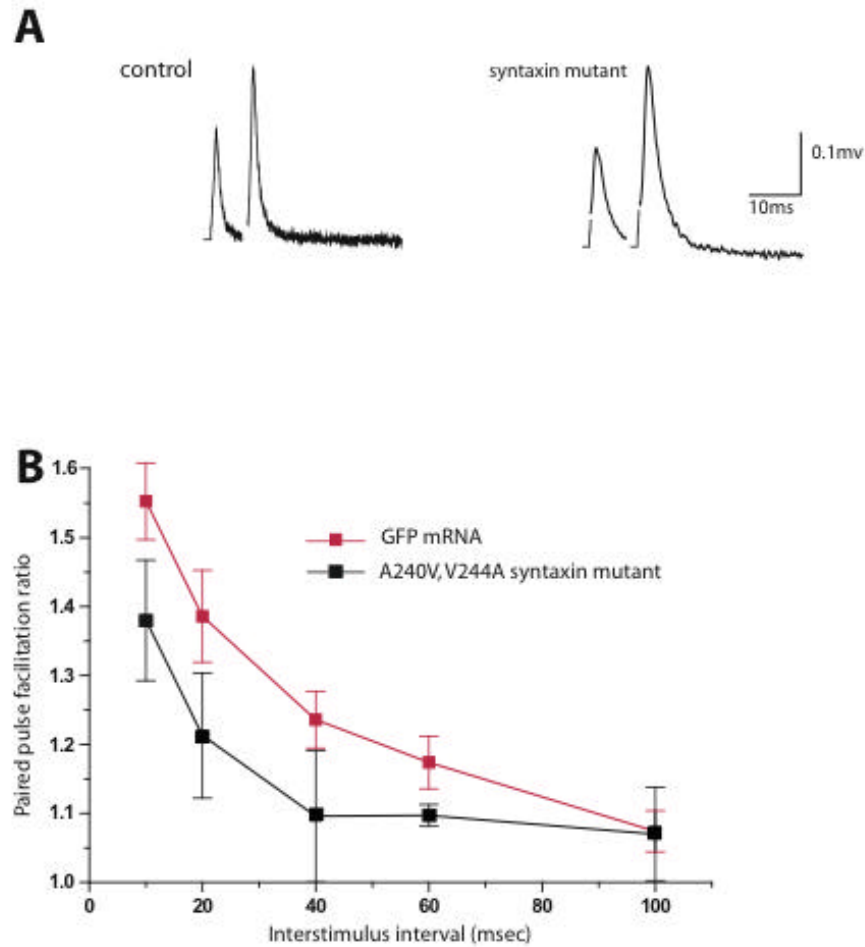


Figure 10: Injection of mutant stx1A (A240V, V244A) into 2-cell stage *Xenopus laevis* embryos results in decreased paired pulse facilitation of EPPs. (A) Representative EPPs; paired pulse facilitation recordings with a 10ms interstimulus interval. Left trace: control animal; Right trace: synprint injected animal. (B) Compiled data; mut. stx1A decreased P.P.F. compared to GFP mRNA injected animals. Control animals represented by red squares (n=15); mutant stx1A (A240V, V244A) animals represented by black squares (n=3).

Figure 10

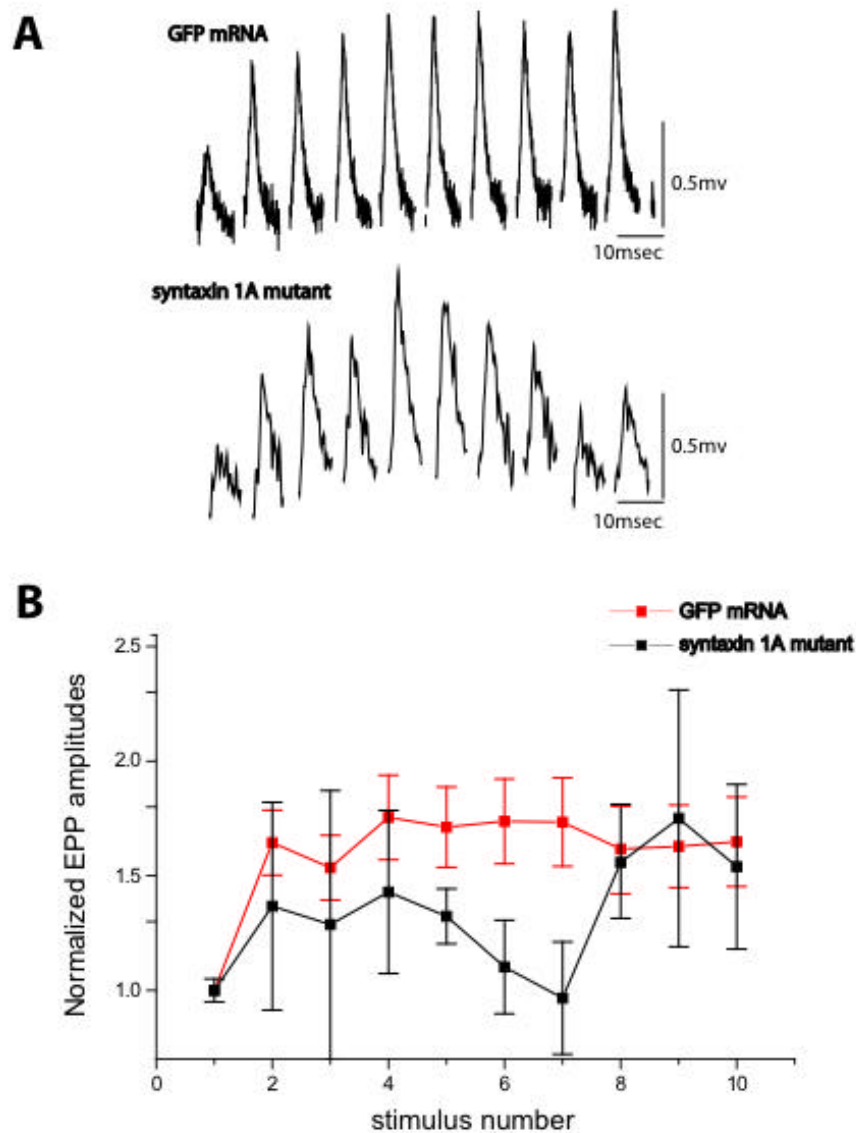


Figure 11: Injection of syntaxin mutant A240V,V244A into 2-cell stage *Xenopus laevis* embryos, tetanic potentiation of EPPs. (A) Representative EPPs; recorded during 100Hz stimulation. top trace: GFP mRNA control animal, average of 10 sweeps; bottom trace: A240V, V244A injected animal, average of 10 sweeps. (B) Compiled data; syntaxin mutant injection shows a trend towards decreased tetanic potentiation. Normalized EPP size during 100Hz stimulation. Control animals represented by red squares (n=6); syntaxin mutant animals represented by black squares (n=2).

Figure 11

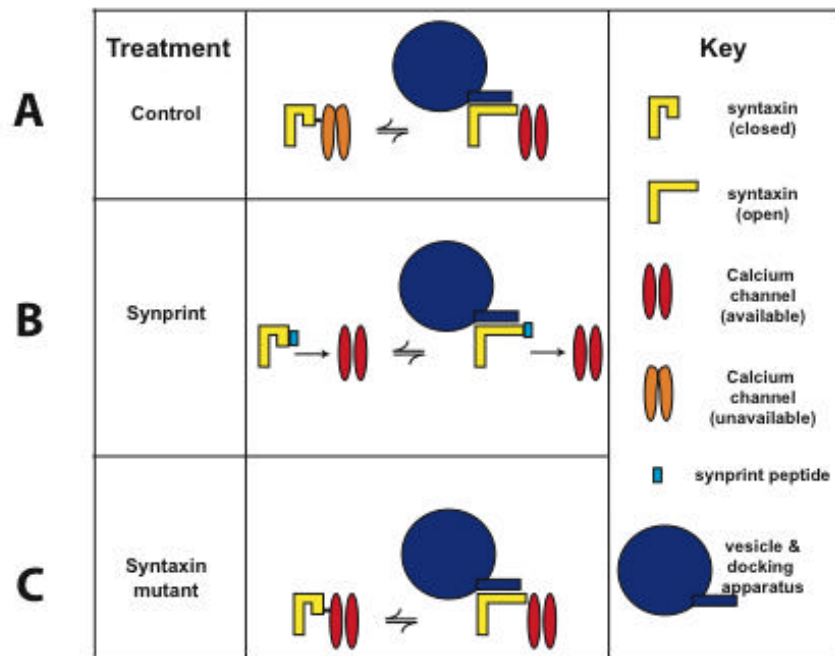


Figure 12: Conceptual representation of protein interactions and implications for release. (A) In this treatment state; active zones have mixed occupied and unoccupied vesicle docking sites. When vesicles are present calcium channels are available for calcium entry; This control state should result in normal NT release. (B) With synprint injection, there is no syntaxin, N-type channel interaction. Therefore all channels are available and channels may drift out of position. NT release is governed by a balance between channel availability and distance between channels and calcium sensors for release. (C) In this state the A240V, V244A mutant can interact structurally with the calcium channel but there is no functional interaction. Thus, calcium channels are constantly in an available state. NT release is predicted to increase due to increased calcium entry.

Figure 12

References

- Aravanis AM, Pyle JL, Tsien RW, (2003) Single synaptic vesicle fusing transiently and successively without loss of identity. *Nature* 423(6940) 643-647.
- Atlas D. (2001) Functional and physical coupling of voltage-sensitive calcium channels with exocytotic proteins: ramifications for the secretion mechanism. (Review) *Journal of Neurochemistry* (77) 972-985.
- Bauer PJ (2001) The local Ca^{2+} concentration profile in the vicinity of a Ca^{2+} channel. *Cell Biochem. Biophys* 35(1): 49-61.
- Bennett MVL, Calakos N, Scheller R (1992) Syntaxin; a Synaptic Protein Implicated in Docking of Synaptic Vesicles at Presynaptic Active Zones. *Science* (257) 255-259.
- Bergsman JB, Tsien RW (2000) Syntaxin Modulation of Calcium Channels in Cortical Synaptosomes As Revealed by Botulinum Toxin C1. *J. Neuroscience* 20 (12): 4368-4378
- Bezprozvanny I, Zhong R, Scheller RH, Tsien RW (2000) Molecular determinants of the functional interaction between syntaxin and N-type Ca^{2+} channels gating. *PNAS* 97 (25): 13943-13948.
- Bezprozvanny I, Scheller RH, Tsien RW (1995) Functional impact of syntaxin on gating N-type and Q-type calcium channels. *Nature* 378 (7): 623-626.
- Chen YA, Scheller RH (2001) Snare-Mediated Membrane Fusion. *Nature Reviews* (2): pg 98-105.
- Cohen R, Elferink L Atlas D (2003) The C2A Domain of Synaptotagmin Alters the Kinetics of Voltage gated Ca^{2+} Channels $\text{Ca}_v1.2$ (Lc-type) and $\text{Ca}_v2.3$ (R-type). *Journal of Biological Chemistry* 278 (11): 9258-9266.

Degtjar VE, Scheller Rh, Tsien RW (2000) Syntaxin modulation of slow inactivation of N-type calcium channels. *J. Neuroscience* 20 (12): 4368-4378.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* (410): 41-49.

Fernandez JM, Neher E, Gomperts BD, Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* 312 (5993) 453-455.

Hayashi T, Mccmahon H, Yamaski S, Binz T, Hata Y, Sudhof T, Niemann H, (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO* 13 (21) 50551-5061.

Heuser JE, Reese TS, Landis DMD (1975) Functional changes in Frog NMJ studied with Freeze Fracture. *J. Neurocytol.* 3: 109-131.

Heuser JE (1989) Review of Electron Microscopic Evidence Favouring Vesicle Exocytosis as the Structural Basis for Quantal Release During Synaptic Transmission. *Q.J. Exp. Physiol.* 74: 1051-1069.

Hodel A, Schafer T, Gerosa D, Burger M (1994) In Chromaffin Cells, the Mammalian Sec1p Homologue is a Syntaxin 1A Binding Protein Associated With Chromaffin Granules. *Journal of Biological Chemistry* 269 (12): 8623-8626.

Inoue A, Obata K, Akagawa K (1992) Cloning and Sequence analysis of cDNA for a Neuronal Cell Membrane Antigen, HPC-1. *Journal of Biological Chemistry* 267 (15): 10613-10619.

Katz B, Miledi R (1967) The Timing of calcium action during neuromuscular transmission. *J. Physiol.* 189 (3): 535-544.

Katz B, Mildei R (1967) Ionic Requirements of Synaptic Transmitter Release. *Nature* 192 (2): 407-436.

Kee Y, Scheller R (1996) Localization of Synaptotagmin-Binding Domains on Syntaxin. *J. NeuroSci.* 16 (6): 1975-1981.

Kee Y, Lin RC, Hsu SC, Scheller RH (1995) Distinct Domains of Syntaxin Are Required for Synaptic Vesicle Fusion Complex Formation and Disassociation. *Neuron* (14): 991-998.

Kuffler (1942) Electrical Potential Changes at an Isolated Nerve-muscle Junction. *J. Neurophysiol.* (5): 211-230.

Li C, Ullrich B, Zhang J, Anderson R, Brose N, Sudhof T (1995) Ca^{2+} dependent and independent activities of neural and non-neural synaptotagmins. *Nature* 375 (15): 594-599.

Llinas R (1982) Calcium in Synaptic Transmission. *Scientific America* 247 (4): 56-65.

Magelby KL (1987) Short-term changes in Synaptic Efficacy. In *Synaptic Function*, Edelman GM, Gall VE, Cowan WM, Eds. New York: John Wiley and Son: p 21-56.

Mochida S, Sheng ZH, Baker C, Kobayashi H, Catterall W (1996) Inhibition of Neurotransmission by Peptides Containing the Synaptic Protein Interaction Site of N-type Ca^{2+} channels. *Neuron* 17: 781-788.

Mochida S, Saisu S, Kobayashi H, Abe T (1995) Impairment of Syntaxin by Botulinum Neurotoxin C, or Antibodies Inhibits Acetylcholine Release But Not Ca^{2+} Channel Activity. *J. Neuroscience* 15 (3) 905-915.

Naraghi N, Neher E (1997) Linearized buffered Ca^{2+} diffusion in microdomains and its implications for calculations of $[\text{Ca}^{2+}]$ at the mouth of a calcium channel. *J. Neuroscience* 17 (18): 6961-6973.

Nieuwkoop PD, Faber J (1967) Normal Table of *Xenopus laevis*. (Daudin) N-Holland, Amsterdam.

O'Connor V, Heuss C, De Bello WM, Dresbach T, Charlton MP, Hunt JH, Pellegrini LL, Hodel A, Burger MM, Betz H, Augustine GJ, Schafer T (1997) Disruption of syntaxin-mediated protein interactions blocks neurotransmitter secretion. PNAS (94) 12186-12191.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M (1989) The Identification of a Novel Synaptosomal-Associated Protein, SNAP-25, Differentially Expressed by Neuronal Subpopulations. J. Cell Biol. 109: 3039-3052.

Pellegrini LL, O'Connor V, Lottspeich F, Betz H (1995) Clostridial Neurotoxins Compromise the Stability of a Low Energy SNARE Complex Mediating NSF Activation of Synaptic Vesicle Fusion. EMBO 14 (19): 4705-4713.

Rettig J, Heinemann C, Ashery U, Sheng ZH, Yokoyama C, Catterall W, Neher E (1997) Alteration of Ca^{2+} Dependence of Neurotransmitter Release by Disruption of Ca^{2+} Channel/Syntaxin Interaction. J. Neuroscience 17 (17) 6647-6656.

Schiavo G, Shone C, Bennett M, Scheller R (1997) Botulinum Neurotoxin Type C Cleaves a Single Lys-Ala Bond within the Carboxyl-Terminal Region of Syntaxins. Journal of Biological Chemistry 270 (18) 10566-10570.

Seidman S, Soreq H (1997) Transgenic *Xenopus*: Microinjection Methods and Developmental Neurobiology. Humana Press Inc. Totowa New Jersey.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S (1997) SNAP receptors implicate in vesicle targeting and fusion. Nature 362: 318-324.

Sollner T, Bennett M, Whiteheart S, Scheller R, Rothman J (1993) A Protein Assembly-Disassembly Pathway In Vitro That May Correspond to Sequential Steps of Synaptic Vesicle Docking, Activation, and Fusion. *Cell* 75: 409-418.

Sorensen J, Matti U, Wei S, Nehring R, Voets T, Ashery U, Binz T, Neher E, Rettig J (2002) The SNARE Protein SNAP-25 is Linked to Fast Calcium Triggering of Exocytosis. *PNAS* 99 (3) 1627-1632.

Stanley EF, Reese TS, Wang GZ (2003) Molecular scaffold reorganization at the transmitter release site with vesicle exocytosis or botulinum toxin C1. *European Journal of Neuroscience*. (18): 2403-2407.

Trimble WS, Cowan DM, Scheller RH (1988) VAMP1: A Synaptic Vesicle-Associated Integral Membrane Protein. *PNAS* (85): 4538-4542.

Walker D, Waard M (1998) Subunit Interaction Sites in Voltage-Dependent Ca^{2+} Channels Role in Channel Function. (Review) *TINS* 21 (4).

Wiser O, Bennett MK, Atlas D (1996) Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L-N-type Ca^{2+} channels. *EMBO* 15 (16): p 4100-4110.

Wu LG, Sun JY (2001) Fast Kinetics of Exocytosis Revealed by Simultaneous Measurements of Presynaptic Capacitance and Postsynaptic Currents at a Central Synapse. *Neuron* 30 (1): 5-7.

Wu M, Fergstad T, Lloyd T, He Y, Broadie K, Bellen H (1999) Syntaxin1A Interacts With Multiple Exocytic Proteins to Regulate Neurotransmitter Release In Vivo. *Neuron* (23): 593-605.

Yazejian B, DiGregorio DA, Vergara JL, Poage RE, Meriney SD, Grinnell AD (1997) Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured *Xenopus* nerve-muscle synapses. *Journal of Neuroscience* 17(9): 2990-3001.

Yokoyama C, Sheng Z, Catterall W (1997) Phosphorylation of the Synaptic Protein Interaction Site on N-type Calcium Channels Inhibits Interactions with SNARE Proteins. *J. Neuroscience* 17 (18): 6929-6938.

Zamponi GW (2003) Regulation of Presynaptic Calcium Channels by Synaptic Proteins. *Journal of Pharmacological Sciences*. (92): 79-83.

Zhang X, Kim_Miller M, Fukuda M, Kowalchuk J, Martin T (2002) Ca^{2+} Dependent Synaptotagmin Binding to SNAP-25 is Essential for Ca^{2+} Triggered Exocytosis. *Neuron* (34): 599-611.

Zhong P, Chen YA, Tam D, Chung D, Scheller RH, Miljanich GP (1997) An α -Helical Minimal Binding Domain Within the H3 Domain of Syntaxin is Required for SNAP-25 Binding. *Biochemistry* (36): 4317-4326.

Zucker RS (1989) Short-term Synaptic Plasticity. *Annu. Rev. Neurosci.* (12): 13-31.