Structural, Genetic and Physiological Analysis of the Juxtamembrane Region of *Drosophila* neuronal-Synaptobrevin

by

Colin Don Malcolm DeMill

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell and Systems Biology
University of Toronto

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Abstract

Synaptic transmission requires the fusion of neurotransmitter containing vesicles with the neuron's plasma membrane in a temporally restrictive manner. In *Drosophila*, this challenge is accomplished in part by the SNARE protein neuronal-Synaptobrevin (n-Syb). The juxtamembrane region of this molecule, linking the cytosolic SNARE motif and transmembrane region, is hypothesized to play a functional role in facilitating membrane fusion. This short, 10 amino acid, segment contains numerous charged residues and one conserved tryptophan residue. Its short rigid structure may be important in transducing force during SNARE complex assembly.

Tryptophan residues, common in membrane proteins, are often observed at the membrane-water interface. It was hypothesized that this conserved tryptophan residue was important for anchoring and positioning n-Syb in the membrane. Proteins produced with tryptophan mutated were tested for anchoring and stability in a membrane model using NMR
spectroscopy. Experiments testing depth of insertion using exposure to oxygen, a paramagnetic species, and exchange with deuterium demonstrated that tryptophan anchored n-Syb in the membrane.

To test a potential functional role for the juxtamembrane region of n-Syb in synaptic transmission, a reverse genetic approach was employed. Wild-type and mutant P-element clones were made using the genomic sequence of n-syb including the endogenous promoter. n-Syb was found to be expressed, integrate and orient correctly in the membrane of Drosophila S2 cells. Transgenic Drosophila, produced via P-element transformation, were also found to produce transgenic protein. Transgenic expression of wild-type n-syb was found to restore an n-syb hypomorphic mutant from severe motor impairment and limited lifespan to wild-type levels. Synaptic transmission was assessed in 3rd instar larval preparations of mutant and wild-type transgenics. Mutation of the tryptophan residue and insertion of a short flexible linker were both found to inhibit synaptic transmission, while insertion of a long flexible linker was not.
Dedication

To Sadie and Sam
"The limits of the possible can only be defined by going beyond them into the impossible."

Arthur C. Clarke
The successful completion of this project would not have been possible without the technical, intellectual and personal support from many people.

My supervisory committee contributed ideas, inspired success and ensured rigorous interpretation of data. Dr. Bryan Stewart, supervisor, contributed imaginative ideas, endless optimism and technical support. It was a wonderful opportunity to work and learn in Dr. Stewart’s laboratory. Dr. William Trimble, committee member, contributed ideas, offered technical assistance and taught the importance of designing experiments in a logical and critical way. Dr. Voula Kanelis, committee member, helped bridge the gap between biology and chemistry, offered technical assistance and new experimental ideas. I would like to thank the committee for thorough review of this work. Dr. Milton Charlton, former committee member, offered countless experimental ideas. Proposal examiners, Dr. Vince Tropepe and Dr. Melanie Wooden, offered ideas and suggestions in helping to direct further experiments.

I would like to acknowledge Dr. David Deitcher of Cornell University, for opening his laboratory to me and teaching me fundamental molecular biological skills and specific techniques for the manipulation of SNARE proteins. Also, Dr. Joel Levine, UTM, was very helpful in interpreting genomic expression data and Dr. Troy Littleton, MIT, provided helpful suggestions.

I would like to thank members of the Stewart lab for endless technical assistance and support during my time in the lab: Dr. Xinping Qiu (cloning work), Marzena Serwin (NMR work), Farah Jazuli (fly work), Nicole Novroski (anatomy), Alanna Bolotta (anatomy), Dr. Sara Seabrooke, Matt Laviolette, Jing Guo Ma, Dr. Marta Kisiel, Dr. Nicola Hains, Ahmed Faress, Eunice Furtado, Abdullah Ishaque, Julieta Lazarte, Kris McKenzie, Alicja Nowicki, Jessica Monteiro, Vanya Pinto, Renuka Ramlogan, Owen Randlett, Rachelle Dinchong, Alexandra Quimby, and Dr. Melissa Massey.

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And most of all, I would like to acknowledge my family for providing support, encouragement and motivation to succeed: Lee, Sadie & Sam.
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<tr>
<td>7S</td>
<td>SNARE complex, based on size observed on a 2 dimensional gel</td>
</tr>
<tr>
<td>20S</td>
<td>SNARE complex associated with NSF and α-SNAP, based on size observed on 2 dimensional gel</td>
</tr>
<tr>
<td>AA</td>
<td>Transgenic constructs with mutations tryptophan 89 and leucine 90 to alanine</td>
</tr>
<tr>
<td>α-SNAP</td>
<td>Soluble NSF attachment protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>bar</td>
<td>Unit of pressure equivalent to 100kPa (kilo pascals)</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>Isotope of carbon (6 protons, 7 neutrons)</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CATCHR</td>
<td>Complex associated with tethering containing helical rods</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cis-SNARE</td>
<td>Fully assembled SNARE complex with transmembrane domains in the same membrane</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxy group</td>
</tr>
<tr>
<td>C terminal</td>
<td>Carboxy terminal of a peptide or protein</td>
</tr>
<tr>
<td>$Cyo$</td>
<td>Curly of Oster (Dominant curly wing phenotype, 2$^{nd}$ chromosome balancer)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPC</td>
<td>Dodecylphosphocholine</td>
</tr>
<tr>
<td>DrPr</td>
<td>Drop (dominant eye phenotype)</td>
</tr>
<tr>
<td>$e$</td>
<td>ebony (recessive adult phenotype: black body)</td>
</tr>
<tr>
<td>EJC</td>
<td>Excitatory junctional current (current measured in muscle fibre)</td>
</tr>
<tr>
<td>EJP</td>
<td>Excitatory junctional potential (depolarization stimulated in muscle fibre)</td>
</tr>
<tr>
<td>ELAV</td>
<td>Embryonic lethal abnormal vision (pan neural driver)</td>
</tr>
<tr>
<td>ELK</td>
<td>Glutamine, leucine, serine and lysine-rich proteins. Also known as: Piccolo/bassoon or Rab-6 interacting protein</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate. Used to produce mutations in DNA</td>
</tr>
<tr>
<td>$^{19}\text{F}$</td>
<td>Fluorine, natural form (9 protons, 10 neutrons)</td>
</tr>
<tr>
<td>F1,2</td>
<td>Familial generation1, familial generation 2</td>
</tr>
</tbody>
</table>
ΔF33B Null mutation of n-syb (imprecise P-element excision)
F33R Near null mutation of n-syb (P-element insertion).
FM7 First multiply inverted-7 (First chromosome balancer - prevents recombination)
Gla Glazed (dominant eye phenotype: glazed eyes)
Gal4/UAS Galactose/upstream activation sequence. Driver of choice produces Gal4 (a yeast transcription activator protein, which then binds to, and activates UAS (attached to the gene of choice to be turned on)
$^1$H Hydrogen, natural form (1 proton, no neutrons)
$^2$H$_2$O Deuterium oxide (composed of hydrogen with 1 proton and 1 neutron)
Habc N terminal domain of syntaxin consisting of 3 α-helices
HIV Human Immunodeficiency Virus
HSQC Heteronuclear single quantum coherence
$I^4$ Hypomorphic mutant of n-syb
KDa Kilo Daltons
$k_B T$ Unit of thermal energy: k: Boltzmann constant, T temperature
L6 Transgenic constructs with 6 amino acid insert
L24 Transgenic constructs with 24 amino acid insert
Ly Lyre (dominant wing phenotype: oar or lyre shaped)
Mini Spontaneous miniature potential (observed in muscle fibre)
mRNA Messenger ribonucleic acid
Munc-13 Active zone protein with MUN domain
Munc-18 SM protein family, binds to Syntaxin
$^{15}$N Isotope of nitrogen (7 protons, 8 neutrons)
NaF Sodium fluoride
NH$_2$ Amino group
NMJ Neuromuscular junction
NMR Nuclear magnetic resonance spectroscopy
NSF N-ethylmaleimide-sensitive factor
$n$-syb neuronal-synaptobrevin (gene)
n-Syb neuronal-Synaptobrevin (protein)
N terminal Amino terminal of a peptide or protein
N-type Ca$^{2+}$ channel associated with the nervous system
O$_2$ Oxygen
OR  Oregon-R (wild-type control line)
p35  Syntaxin
p65  Synaptotagmin
pBS  Plasmid - blue script
PC12  Pheochromocytoma cells (rat cell culture line)
PCR  Polymerase chain reaction
pCaSpeR2  Plasmid - used for P-element insertions, encodes white gene
pET15b  Plasmid with poly His tag, used to produce protein for NMR experiments
PMP1  38 residue plasma membrane protein from Yeast
ppm  Parts per million
pOT2  Plasmid used to produce cDNA clone for NMR constructs
Q-SNARE  SNARE protein with glutamine at the zero layer
Rab-3  Ras family of monomeric G proteins
RIM  Rab-3 interacting molecule (organizer of the active zone)
RNA  Ribonucleic acid
RRP  Readily releasable pool (of neurotransmitter vesicles)
R-SNARE  SNARE protein with arginine at the zero layer
S2  Schneider-2 cell (Drosophila neural progenitor cell culture line)
Sb  Stubble (dominant adult phenotypic marker: stubble bristles)
SIIS  Solvent induced isotope shift
SIV  Simian Immunodeficiency Virus
SM protein  Sec-1/Munc-18-like family
SNAP-25  Synaptosomal associated protein of 25 kD
SNARE  Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNAREpin  trans-SNARE complex resembling a hair pin
SRP  Signal recognition particle
Syx  Syntaxin
Tb  Tubby (Dominant larval phenotypic marker: fat, round larva and pupa)
TM2  Third multiply inverted-2 (3rd chromosome balancer - prevents recombination)
TM6  Third multiply inverted-6 (3rd chromosome balancer - prevents recombination)
trans-SNARE  Partially assembled SNARE complex with transmembrane domains in opposite membranes
t-SNARE  SNARE protein associated with the target membrane
*Ubx*  Ultrabithorax (dominant adult phenotype - bi-thorax)

**V5**  Epitope tag

**VAMP**  Vesicle associated membrane protein

**v-SNARE**  SNARE protein associated with the vesicle membrane

**w**\(^+\), **w**\(^-\)  Presence or absence of white gene (encodes red eyes)

**w**\(^{1118}\)  white gene

**WT**  Wild-type transgenic constructs

**Y**  Y chromosome

### Table 1: Amino Acid Single Letter Code (for sequences > than 5 residues)

<table>
<thead>
<tr>
<th>A</th>
<th>alanine</th>
<th>M</th>
<th>methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>cysteine</td>
<td>N</td>
<td>asparagine</td>
</tr>
<tr>
<td>D</td>
<td>aspartic Acid</td>
<td>P</td>
<td>proline</td>
</tr>
<tr>
<td>E</td>
<td>glutamic acid</td>
<td>Q</td>
<td>glutamine</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine</td>
<td>R</td>
<td>arginine</td>
</tr>
<tr>
<td>G</td>
<td>glycine</td>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>H</td>
<td>histidine</td>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>I</td>
<td>isoleucine</td>
<td>V</td>
<td>valine</td>
</tr>
<tr>
<td>K</td>
<td>lysine</td>
<td>W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>L</td>
<td>leucine</td>
<td>Y</td>
<td>tyrosine</td>
</tr>
</tbody>
</table>
List of Constructs and Drosophila Stocks

Constructs for *in vitro* Biophysical Analysis (*pET15b*)

- wt-TM-n-Syb
- AA-TM-n-Syb
- WW-TM-n-Syb

Genomic Constructs

- pCaSpeR2-*n-syb*$_{WT}^{W}$
- pCaSpeR2-*n-syb*$_{WW}^{W}$
- pCaSpeR2-*n-syb*$_{AA}^{A}$
- pCaSpeR2-*n-syb*$_{WT}^{16}$
- pCaSpeR2-*n-syb*$_{WT}^{124}$

Drosophila Stocks

- *w*$_{1118}^{1118}$
- *Oregon-R*
- *n-syb*$_{AF33B}^{A}$
- *n-syb*$_{14}^{14}$
- *n-syb*$_{F33R}^{F}$
- UAS *n-syb* RNAi / ELAV Gal4
- Gla/Cyo
- TM2Ubx/TM6CSb
- Sp/Cyo;Ly/TM6SbTb
- FM7;Ly/TM6SbTb
- DrPr/ TM6SbTb
- Df(3L)BSC800, TM6Sb (Bloomington #27372)

X Chromosome transgenic insertions

- *n-syb*$_{WT}^{WT 11H}$
- *n-syb*$_{WT}^{WT 16A}$
- *n-syb*$_{WT}^{WT 45H}$
2<sup>nd</sup> Chromosome transgenic insertions

- n-syb<sup>WT 45I</sup>
- n-syb<sup>L24 24A</sup>
- n-syb<sup>L24 45A</sup>
- n-syb<sup>WT 11A</sup>
- n-syb<sup>WT 11J</sup>
- n-syb<sup>WT 15A</sup>
- n-syb<sup>WT 27A</sup>
- n-syb<sup>WT 27C</sup>
- n-syb<sup>WT 40A</sup>
- n-syb<sup>WT 40B</sup>
- n-syb<sup>WT 45C</sup>
- n-syb<sup>WT 45J</sup>
- n-syb<sup>WT 54A</sup>
- n-syb<sup>WT 54B</sup>
- n-syb<sup>WW 7A</sup>
- n-syb<sup>WW 7B</sup>
- n-syb<sup>WW 35B</sup>
- n-syb<sup>WW 46A</sup>
- n-syb<sup>AA 2B</sup>
- n-syb<sup>AA 4A</sup>
- n-syb<sup>AA 4B</sup>
- n-syb<sup>AA 5B</sup>
- n-syb<sup>AA 15B</sup>
- n-syb<sup>AA 19B</sup>
- n-syb<sup>AA 27A</sup>
- n-syb<sup>AA 32A</sup>
- n-syb<sup>AA 40A</sup>
- n-syb<sup>AA 40B</sup>
- n-syb<sup>AA 45A</sup>
- n-syb<sup>AA 60A</sup>
- n-syb<sup>AA 60B</sup>
n-syb<sup>L6 9A</sup>
n-syb<sup>L6 23A</sup>
n-syb<sup>L24 14A</sup>
n-syb<sup>L24 29A</sup>
n-syb<sup>L24 32B</sup>
n-syb<sup>L24 40A</sup>
n-syb<sup>L24 44B</sup>
n-syb<sup>L24 55A</sup>
n-syb<sup>L24 57A</sup>

3<sup>rd</sup> Chromosome transgenic insertions

n-syb<sup>WT 11F</sup> (no recombinant lines)
n-syb<sup>WT 11G</sup> (5 recombinant lines)
n-syb<sup>WT 45E</sup> (2 recombinant lines)
n-syb<sup>WT 57F</sup> (no recombinant lines)
n-syb<sup>WT 59A</sup> (9 recombinant lines)
n-syb<sup>WT 59B</sup> (4 recombinant lines)
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Chapter 1 Introduction

Neural transmission

An organism's ability to respond to and interact with external stimuli is facilitated by the nervous system. In its simplest form, information is received by a sensory or afferent neuron, conducted to an interneuron in the central nervous system for processing, then conducted via a motor or efferent neuron to facilitate a response to the stimulus. In its most complex form, higher order processing in the central nervous system allows high order functions such as navigation, language and consciousness. Whether a simple reflex arc or a high level cortical function, all of these processes involve communication between individual neurons via chemical transmission across the synaptic cleft. Chemical transmission between neurons is fundamental to all nervous systems from the simplest such as the nerve net found in the jellyfish to the most complex such as the primate brain. Chemical transmission requires the exocytosis of neurotransmitter containing vesicles to occur in a highly efficient and controlled manner (Squire et al., 2003).

Within neurons, signals are propagated electrically. Typically, passive potentials conduct through the dendritic arbor to the neuron's cell body. Here, if a sufficient threshold voltage is achieved, an active, action potential, may be produced which would then conduct down the axon. Upon reaching the neuron's synaptic varicosities or terminals where specialized active zones are found, chemical synaptic transmission may occur (Squire et al., 2003). The active zones contain an aggregation of channels, proteins and vesicles specialized for neurotransmitter vesicle exocytosis (Dreyer et al., 1973; Heuser & Reese, 1973). This region of the neuron is also associated with an electron dense postsynaptic density on the target neuron or muscle marking the location of postsynaptic receptors (Schoch & Gundelfinger, 2006). Separating the two structures marking the pre and post synapse is the narrow synaptic cleft between the two cells (Squire et al., 2003; see Figure 1 for an example of the Drosophila neuromuscular junction).
Figure 1: *Drosophila* Neuromuscular Junction

Schematic depicting the *Drosophila* 3rd instar larval preparation, ventral side down, filleted and pinned, brain intact (green). Note the characteristic pattern of muscle fibres repeated in each segment. One nerve, containing multiple axons, reaches each hemi-segment. The first expansion depicts the neuromuscular junction (NMJ) on muscles 6/7 stained with a fluorescent neural stain (anti-HRP conjugated to FITC). The second expansion depicts the
vesicle cycle in a synaptic terminal bouton. Vesicles in the readily releasable pool (RRP) are primed and ready for fusion at the active zone. Fusion releases neurotransmitter into the synaptic cleft. After fusion, vesicles are recycled, refilled and enter the recycling pool. During normal sustained activity, vesicles move from the recycling pool to the RRP and complete the cycle. During higher levels of activity, vesicles move from the reserve pool to the recycling pool. Note, in reality, the vesicle pools are not anatomically distinct as shown in this simplified schematic.
Typically, the action potential's invasion of this region triggers voltage-gated calcium channels to open, allowing $\text{Ca}^{2+}$ to enter into the active zone. With a high concentration of $\text{Ca}^{2+}$ outside the cell, a large electrochemical gradient is established, driving $\text{Ca}^{2+}$ into the cell. Calcium ions then bind to Synaptotagmin, a protein associated with both $\text{Ca}^{2+}$ channels and a complex linking neurotransmitter containing vesicles with the neuron's membrane (Figure 2). The proteins that make up this complex are essential in facilitating the neurotransmitter vesicle's membrane to fuse with the neuron's plasma membrane and allow transmitter to diffuse into the synaptic cleft. The entire process, from action potential invasion to opening of channels in the postsynaptic receptors occurs in $<1\text{ms}$, requiring that all structures involved in vesicle fusion to be located in close proximity to $\text{Ca}^{2+}$ channels (review by Schneggenburger et al., 2012). Early work by Fatt & Katz (1952) on the frog neuromuscular junction revealed that postsynaptic events are quantal in nature. The vesicle hypothesis followed with the idea that neurotransmitter was packed into vesicles and thus released in discrete packets (Palay et al., 1956). In addition to vesicle fusion triggered by action potentials, spontaneous fusion events are also observed and termed miniature potentials (Fatt & Katz, 1952). Modern work has revealed that these events likely occur from two distinct mechanisms, one that is $\text{Ca}^{2+}$ dependent and one that is $\text{Ca}^{2+}$ independent (Capogna et al., 1996). One final form of spontaneous vesicle fusion is observed to occur in some systems immediately after action potential evoked release and is termed asynchronous release (Goda and Stevens, 1994). But solving exactly how neurotransmitter is released from vesicles into the synaptic cleft has proven to be an intriguing problem.
Figure 2: Molecular structure of vesicle fusion machinery

Schematic of the major proteins involved in vesicle docking and fusion for Ca^{2+} evoked neurotransmitter release. The synaptic cleft separates the presynaptic motor neuron terminal's active zone from a postsynaptic muscle fibre. The synaptic vesicle is filled with glutamate (pink dots) which, upon fusion of the vesicle membrane with the plasma membrane, will diffuse into
the synaptic cleft and bind to postsynaptic glutamate receptors. Vesicles are docked at the active zone via binding of vesicle associated Rab-3 (beige) to the active zone organizer RIM (Rab-3 Interacting Molecule; light blue). RIM also binds to Munc-13 (orange) and Ca$^{2+}$ channels (yellow). Munc-13 binds to Munc-18 (grey) which interacts with the Habc domain of the plasma membrane SNARE protein Syntaxin (red). Other SNARE proteins include SNAP-25 (dark green) which associates with the plasma membrane via palmitoylation and n-Syb (VAMP; dark blue) bound to the vesicle. This schematic depicts the SNARE complex beginning to form between the three SNARE proteins which align in parallel from membrane distal N termini to membrane proximal C termini. A 4-helix coiled-coil forms with n-Syb/VAMP providing one helix, Syntaxin one helix and SNAP-25 two helicies. Full assembly or zipperring of the SNARE complex may be prevented by Complexin (light green). One other vesicle protein, the calcium binding protein Synaptotagmin (purple), is depicted. It binds Ca$^{2+}$ to its C2A and C2B domains and associates with the plasma membrane, Ca$^{2+}$ channels and the SNARE complex. Upon Ca$^{2+}$ entry, Synaptotagmin may undergo a conformation change which relieves a block preventing SNARE complex assembly such as Complexin. The SNARE complex then fully assembles and membrane fusion occurs. Not depicted for clarity are α-Liprin (binds to RIM) and RIM Binding Protein (also binds RIM to Ca$^{2+}$ channels). Other active zone proteins not depicted include scaffolding proteins such as the large cytomatrix proteins piccolo/bassoon and the Rab-6 interacting proteins ELKS. Adapted from: Sudhof & Rizo (2011); Kaeser et al., 2011; Fasshauer et al., (1998).
The discovery of SNARE Proteins

The seemingly simple process of fusion of neurotransmitter containing vesicles with the cell’s plasma membrane would be impossible if there was no method of overcoming the biophysical forces repelling these membranes (Chernomordik & Kozlov, 2003). A set of three proteins, which are members of the SNARE family of proteins, have been demonstrated to be essential to this process (Weber et al., 1998). The acronym SNARE, which stands for soluble N-Ethylmaleimide-sensitive-factor attachment protein receptor, evolved from a series of experiments described below.

Classical experimentation on SNARE proteins began long before their discovery and characterization with investigations of the effects of the clostridial toxins associated with tetanus and botulism (Martin, 1892; Hewlett, 1895; Flexner & Noguchi, 1906; Dickson & Shevky, 1923). These toxins arise from the bacteria *Clostridium tetani* (tetanus toxin) and *Clostridium botulinum* (botulism toxin A, B, C₁, C₂, D, E, F & G) respectively and are the most deadly toxins known (Arbuthnot, 1978). This profound toxicity indicated a significant physiological target upon which the toxins exerted their effect. While the physical effects of the toxins differ, with tetanic contraction associated with tetanus toxin and paralysis associated with the botulinum toxins, the ultimate mechanism is the same. SNARE proteins associated with neural transmission are cleaved (Schiavo et al., 1992). The disparate physical presentation is associated with the populations of neurons targeted by the toxins. Motor neurons are targeted by botulinum toxins leading to paralysis, while inhibitory interneurons are targeted by tetanus toxin, leading to tetanic contraction (Link et al., 1992). Early reports described loss of inhibition in the spinal column during tetanus induced from plant toxins (Ringer & Murrell, 1877). While the physiological impact of clostridial toxins on neural transmission was described long ago, the proteins that they act upon and the physiological role that these proteins play was not described until recently.

The observation that vesicle transport within cells was inhibited by the drug N-Ethylmaleimide (Glick & Rothman, 1987) led to the discovery of a protein which plays a integral role in the vesicle cycle termed N-Ethylmaleimide-sensitive factor (NSF) (Block et al., 1988). The hexameric ATPase was originally thought to mediate docking and membrane
fusion (Malhotra et al., 1988; Sollner et al., 1993), but later observed to function in disassembly of fusion machinery for recycling (Rice & Brunger, 1999). It was observed that NSF required other factors found in cytosolic extracts for its role in vesicle trafficking and those factors were thus termed soluble NSF attachment proteins (SNAPs). One such factor, found to bind to NSF and required for its function was α-SNAP, a 35 KDa protein (Clary et al., 1990). The ability of NSF and its soluble attachment protein to bind other proteins involved in fusion was used to isolate and characterize these proteins from brain extracts. Proteins with which the NSF complex bound were thus termed Soluble NSF Attachment REceptors or SNAREs (Rothman & Warren, 1994).

A series of clever experiments by several laboratories utilized cloning and immunological techniques to discover and characterize the three core SNARE proteins essential for neurotransmitter vesicle fusion. The first SNARE protein described, vesicle associated membrane protein 1 (VAMP1), was isolated from the electric ray *Torpedo californica* (Trimble et al., 1988). The electric organ from the fish provided a concentration of synaptic vesicles which could be purified to study vesicle associated proteins. From this preparation, Trimble et al., (1988), created an RNA library which they screened with an antiserum produced against the vesicles. Their screen isolated one clone encoding a small, 120 amino acid protein, which they termed VAMP1. This protein was hypothesized to play a role in vesicle transport and / or membrane fusion. Subsequent work demonstrated two proteins, homologous to *T. californica* VAMP1, expressed in rat brain and were termed VAMP1 and VAMP2 (Elferink et al., 1989). Differential expression of the two VAMPs in rat brain was observed (Trimble et al., 1990; Trimble, 1993). Work from Baumert et al., (1989), described a synaptic vesicle associated protein of 18 KDa in the rat. Due to the proteins synaptic localization and small size it was termed Synaptobrevin but was in fact functionally equivalent to the neural VAMP previously described from *Torpedo* (Trimble et al., 1988). Elaboration of the conserved nature of this protein was demonstrated by Sudhof et al., (1989). VAMP/Synaptobrevin was purified from homogenized rat synaptic vesicles via immunoprecipitation. Oligonucleotides were designed from the extracted proteins and used to isolate matching cDNA clones from a bovine cDNA library. The bovine homolog was found to be identical to the murine version and highly similar to *Torpedo*. Next, a cDNA clone was isolated from a *Drosophila* head cDNA library using a probe from the bovine VAMP.
Comparison of the bovine, *Torpedo* and *Drosophila* sequences revealed homologous proteins with a variable N terminal region, a highly conserved 63 amino acid central region (with 75% identical residues), a conserved putative transmembrane region and an intravesicular tail observed in the *Drosophila* protein. The *Drosophila* VAMP observed by Sudhof *et al.*, (1989) was later found to be expressed broadly, but was not the predominant neural VAMP. This was demonstrated later by DiAntonio *et al.*, (1993) with the identification of neuronal-Syanapto brevin. Overall, these findings implicated VAMP/Synaptobrevin as a central component responsible for neurotransmitter release across species.

The discovery of the second SNARE protein is attributed to Oyler *et al.*, (1989), although previous descriptions of a similar protein termed Polypeptide 20 or Superprotein have been reported in studies of axonal development (Willard *et al.*, 1974; Hess *et al.*, 1992). In an effort to examine expression patterns of mRNA in distinct populations of neurons, a cDNA library was created from Poly(A)⁺ RNA extracted from mouse brain homogenates (Brinks & Wilson, 1986). The library was screened for neuron specific mRNA and cDNA clones were produced to use as probes for *in situ* hybridization. Oyler *et al.*, (1989), used one of these probes to isolate mRNA from rat hippocampal neurons. Antiserum raised against this cDNA allowed the identification and characterization of a 206 residue, 23.3 KDa, neural protein termed Synaptosomal Associated Protein of 25 KDa (SNAP-25). This acronym is not to be confused with the previously mentioned α-SNAP (soluble NSF attachment protein). Initial sequence analysis revealed a largely hydrophilic protein with a suspected amphipathic helix hypothesized to interact with other proteins or membrane components. The protein was found to be expressed in presynaptic nerve terminals associated with postsynaptic specializations and not localized to dendrites or cell bodies. Association with the membrane was hypothesized via a palmitic acid anchor covalently linked to central cystine residues (Hess *et al.*, 1992). SNAP-25 was also observed to exist in 2 isoforms (Bennett *et al.*, 1992). Later work implicated SNAP-25 in vesicle exocytosis because of the observation that inhibition of SNAP-25 expression restricted development of rat cortical neurons (Osen-Sand *et al.*, 1993). SNAP-25 was shown to be highly conserved across species. A cDNA library produced from head homogenates from the fruit fly, *Drosophila melanogaster*, was tested using a probe produced from chicken SNAP-25 and revealed a conserved homolog (Risinger *et al.*, 1993). Using the same technique, the authors also found a homolog from a cDNA
library produced from optic lobe homogenates of the fish, *Torpedo*. The observation of conservation of this protein among both vertebrate and invertebrate species, along with synaptic localization and association with other SNARE proteins (Sollner et al., 1993), indicated a conserved role for SNAP-25 in membrane docking and/or fusion.

The third SNARE protein responsible for neurotransmitter vesicle fusion was characterized by screening binding partners of another synaptic protein: p65/Synaptotagmin. Antibodies against p65 were used to isolate p65 and potential binding partners from synaptic vesicle enriched rat brain homogenate via immunoprecipitation (Bennett et al., 1992). One 35 KDa protein was extracted and used to make an oligonucleotide probe to screen a rat brain cDNA library which produced two proteins: p35A and p35B. Initial characterization demonstrated a highly charged molecule with a putative C terminal membrane anchor similar to VAMP. Further investigation demonstrated that p35 was a membrane protein localized not to vesicle, but rather cell membrane at sites of vesicle fusion. The protein interacted directly with the vesicle associated protein p65 and indirectly with N-type calcium channels (corroborated by Yoshida et al., 1992). p35 was also phosphorylated, indicating a potential role in neural plasticity. Taken together, Bennett et al., (1992), hypothesized that p35 was important for docking vesicles at the membrane and for fusion. They named the protein Syntaxin after the Greek word: συνταξισ, meaning "putting together in order". Based on findings that homologous proteins in yeast were required for vesicle trafficking (Hardwick & Pelham, 1992) and that Syntaxin bound to the NSF-α-SNAP complex (Sollner et al., 1993), Bennett et al., (1993), hypothesized that Syntaxins function as specific target receptors for the regulation of vesicle trafficking.

The evolution of the SNARE hypothesis

Interestingly, these SNARE proteins, from both vesicle and target membrane, bound to the aforementioned NSF-α-SNAP complex from solubilized brain extract which implicated them all in roles such as trafficking, docking and fusion (Rothman & Warren et al., 1994; NSF-α-SNAP-SNAREs termed the 20S particle). These observations led to the SNARE hypothesis: unique vesicle associated SNAREs (or v-SNAREs) associate with unique SNAREs in the target membrane (or t-SNAREs) to enable regulated vesicle trafficking.
between membrane compartments in cells (Bennett & Scheller, 1993; Sollner et al., 1993). And, because homologous proteins were discovered in yeast (Hardwick & Pelham, 1992), this would represent a conserved mechanism for regulating vesicular traffic across Eukaryotes. Such a mechanism would likely require unique SNARE proteins for each trafficking step separating intracellular membranous compartments and indeed, multiple versions of three main SNARE proteins do exist. For example there are 15 homologous versions of Syntaxin in humans and some with several isoforms. While Synaxin 1A and 1B are associated with fusion of neurotransmitter containing vesicles, Syntaxin 5 is associated with the endoplasmic reticulum/Golgi boundary, Syntaxin 7, 8, 12/13 the endosome and Syntaxin 17 and 18 the endoplasmic reticulum (Review by Teng et al., 2001). However, later research undermined the SNARE hypothesis by showing that SNAREs alone could not account for regulated vesicle trafficking. First, by modulating expression levels of SNARE proteins in the yeast, S. cerevisiae, it was discovered that one v-SNARE responsible for trafficking between the Golgi and pre vacuolar organelles via a specific t-SNARE also bound to a different cis-Golgi t-SNARE (von Mollard et al., 1997). Second, Yang et al., (1999), tested 21 combinations of 11 different SNARE molecules and found that viable SNARE complexes formed in all cases. Third, Fasshauer et al., (1999), found no difference in the biophysical properties of SNARE complexes formed from combinations of 4 different Syntaxins and 2 different Synaptobrevins. While these results argue strongly against a diverse array of SNARE proteins responsible for sole regulation of cell trafficking, they do not preclude other factors from interacting with non conserved regions of different SNARE proteins to direct traffic in the cell (Brunger, 2000).

An emerging role for SNARE proteins in exocytosis

A clear role for SNARE proteins in neural exocytosis began to emerge from research in the mid 1990's. At this point the potent physiological effects of clostridial toxins could be connected to the molecules that they cleaved: VAMP (tetanus toxin, botulism toxin B, D, F and G), SNAP-25 (botulism toxin A and E) and Syntaxin (botulism toxin C1) (Link et al., 1992; Schiavo et al., 1992; Schiavo et al., 1994a; review Schiavo et al., 1994b; review by Niemann et al., 1994). Cleavage of VAMP/Synaptobrevin via transgenic expression of
tetanus toxin in *Drosophila* completely eliminated evoked neural transmission demonstrating a clear role for this protein in regulated neural exocytosis (Sweeney *et al*., 1995; Broadie *et al*., 1995). Interestingly, vesicles appeared to cluster normally at active zones supporting the idea that VAMP/Synaptobrevin is not required for targeting and docking contrary to the SNARE hypothesis. Mutants, null for the *Drosophila* VAMP, neuronal-Synaptobrevin (n-syb), also demonstrated elimination of evoked neural transmission from embryo preparations (Deitcher *et al*., 1998). Genetic manipulation of *Drosophila* by Schulze *et al*., (1995), led to the production of a null mutant for Syntaxin (syx). The mutation was embryonic lethal and recordings from embryos produced no evoked or constitutive exocytosis at the neuromuscular junction. Cleavage of SNAP-25 by clostridial toxins applied to rat hippocampal neurons demonstrated reduced probability of evoked release (Capogna *et al*., 1997). A temperature sensitive *Drosophila* mutant of SNAP-25, produced by Rao *et al*., (2001), exhibited a 60% reduction in evoked release at the non permissive temperature. Together, these results clearly demonstrate a role for SNARE proteins in neurotransmitter release downstream of vesicle accumulation at the active zone.

The association of SNARE proteins to form the SNARE complex

The mechanism by which SNARE proteins contribute to membrane fusion was determined through a series of biochemical, biophysical and physiological studies. Sollner *et al*., (1993), demonstrated that SNARE proteins (SNAP receptors) assembled into a complex (Figure 3). The complex was found to be thermally stable and resistant to denaturation by SDS (Hayashi *et al*., 1994). A conserved region of 60 amino acids, demonstrated in VAMP/Synaptobrevin early on (Sudhof *et al*., 1989), was found to be a general feature of SNARE proteins and thus termed the 'SNARE domain' (Weimbs *et al*., 1997). The complex could be observed as 4 X 14nm rods via electron microscopy (Hanson *et al*., 1997), and was ultimately resolved to 2.4Å via X-ray crystallography (Sutton *et al*., 1998). Here it was observed that the SNARE complex resulted from an association between the SNARE motifs of the three SNARE proteins: Syntaxin, SNAP-25 and VAMP. Both VAMP and Syntaxin contribute one alpha helix while SNAP-25 contributes 2 to form a 4 helix coiled-coil of 120Å
(12nm) in length. The proteins associate with a heptad repeat pattern forming 16 layers (Weimbs et al., 1998) numbered -7 from the most membrane distal layer to +8, the most membrane proximal layer (Fasshauer et al., 1998; Sutton et al., 1998). The central zero layer is composed of 3 glutamine (Q) residues from SNAP-25 and Syntaxin and one arginine residue (R) from VAMP. Because this is a general feature of SNARE proteins allowing the discrimination of vesicle from plasma membrane SNAREs, Fasshauer et al., (1998), suggested reclassifying the proteins as Q or R SNAREs depending on the residue at the zero layer. Sutton et al., (1998), suggested that this layer may be the point at which the NSF-α-SNAP complex begins disassembly of the SNARE complex though exposure of the ionic layer to the solvent and thereby destabilizing the heterotrimeric complex. The proteins are arranged in parallel from N to C termini with the 2 helices of SNAP-25 connected by a 54 residue loop. The radius of the coiled-coil varies along its length depending on the side chains associated with residues forming a particular layer. The complex differs from a classic leucine zipper with variation in residues forming the layers (Sutton et al., 1998). The partially assembled SNARE complex is termed 'trans' before full assembly with the proteins in opposite membranes and 'cis' after assembly when the proteins occupy the same membrane. The trans-SNARE complex is also referred to as a 'SNAREpin' in reference to a hair pin (Weber et al., 1998). With the SNARE complex solved, biochemical and physiological experiments were performed to determine its precise role in fusion.

Weber et al., (1998), reconstituted SNARE proteins in liposomes and measured membrane fusion with a variety of techniques. First, because assembled SNARE proteins are resistant to proteolytic cleavage by clostridial toxins, resistance to cleavage would indicate assembly of SNARE complexes. Indeed, VAMP2 containing liposomes, which were easily cleaved by clostridial toxins alone, were resistant when mixed with liposomes containing SNAP-25 and Syntaxin. Next, different fluorophores, bound to lipids, were incorporated into the v- SNARE liposome in a ratio designed to produce a quenched signal. Fusion of the v- and t-SNARE liposomes would result in an increase in fluorescence as lipids became less concentrated. Fusion was observed between v- and t-SNARE liposomes with a half time of approximately 10 minutes at physiological temperatures. This led the authors to postulate that the three SNARE proteins are the minimal components necessary for membrane fusion (Weber et al., 1998). Interpretation of structural data by other authors led to the following
Figure 3: SNARE complex

Assembly of the SNARE complex from amino (N) to carboxy (C) termini between n-Syb / VAMP2 (vesicle, blue), Syntaxin (cell membrane, red) and SNAP-25 (cell membrane, green) leading to vesicle fusion with cell membrane (A). A ribbon diagram of the assembled SNARE complex, a 4 helix coiled-coil, is depicted in (B) with VAMP2 (blue), Syntaxin 1A (red) and SNAP-25

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<tr>
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<td>R. nor.</td>
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(green). The figure was reproduced in Rasmol using data from Ernst and Brunger (2003) (PDB file: 1N7S; crystal structure at 2.4 Å resolution). The single letter code for the protein sequence comprising the SNARE complex is depicted in (C). The 16 layers of contact between SNARE proteins are depicted in red and the residue number completing that portion of the SNARE complex (adapted for mammalian proteins) is indicated. Mammalian sequences depicted are adapted from Fasshauer et al., (1998) while *Drosophila* sequences were obtained from the National Center for Biotechnology Information and aligned based on homology and predictions from Fasshauer et al., (1998).
suggestions regarding how SNARE proteins may promote fusion: first, basic charges at the C
terminal, membrane proximal end of the SNARE complex may promote fusion through
electrostatic interactions with the membrane; second, flexibility in the putative
transmembrane alpha helicities may allow strain, imposed during complex assembly, to distort
membranes and promote fusion; and third, free energy released during complex assembly
may be used in promoting membrane fusion (Sutton et al., 1998).

The lifecycle of the vesicle: docking, priming, fusion
and recycling

Having determined the core molecules for fusion of neurotransmitter vesicles, the next
step was to determine the exact mechanisms of how these and other molecules participate in
the vesicle's lifecycle: docking to the active zone, priming for fusion, membrane fusion and
finally recycling (Brunger, 2000). The first step in docking vesicles to synaptic terminals is
thought to involve tethers. These filamentous structures, visible by electron microscopy
(Siksou et al., 2009), may connect vesicles to cytoskeletal elements such as actin and each
other (review by Shupilakov et al., 2011; see Figure 2). Synapsins are candidate vesicle
proteins which may act as tethers because knockout mice lacking synapsin demonstrated
physiological deficits and reduced vesicle clustering (Li et al., 1995), however other
molecules may be involved (Shupilakov et al., 2011). Vesicles bind to tethering factors via
Rab-3, a small vesicle associated GTPase (Ras family of monomeric G-proteins; Brocker et
al., 2010). Upon reaching the active zone, the vesicles bind to the central organizer RIM
(Rab-3 Interacting Molecule; Wang et al., 1997) via Rab-3-GTPase (Sudhof & Rizo, 2011).
RIM also binds: Munc-13, Ca^{2+} channels which it tethers to the active zone and possibly
Synaptotagmin. Also bound to RIM are α-Liprin, RIM Binding Protein (binds to Ca^{2+}
channels as well) and the ELKs (glutamine, leucine, serine and lysine-rich proteins;
Piccolo/bassoon; Rab-6 interacting protein) (Sudhof & Rizo, 2011). Munc-13, a member of
the CATCHR family of proteins (Complex Associated with Tethering Containing Helical
Rods) is essential to vesicle docking and priming (Neto & Gould, 2011). The protein binds
Ca^{2+} and associates with RIM, Munc-18 and Syntaxin (Jahn & Fasshauer, 2012). Deletion of
the gene for Munc-13 results in severe disruption to vesicle docking observed by electron
microscopy (Siksou et al., 2009) and loss of vesicle fusion (Augustin et al., 1999), but the exact mechanisms are currently unknown (Jahn & Fasshauer, 2012).

Once docked, the vesicle proceeds through a series of priming or maturation steps prior to fusion. The exact mechanism for each process and the final state that the vesicle reaches before Ca\(^{2+}\) entry triggers fusion is still being elucidated. Munc-18, an SM-protein, forms a complex with the Habc domain of Syntaxin, which seems to prevent fusion. Interestingly, deletion of the gene for Munc-18 inhibits neuronal exocytosis implicating Munc-18 in a dual role: first, preventing SNARE complex assembly; second, assisting with and directing SNARE complex assembly by promoting nucleation and/or zippering (Toonen & Verhage, 2007). Munc-13, important for docking, also interacts with Munc-18 and has been implicated to play a role in the priming process as well (Augustin et al., 1999). Interaction with the organizing protein RIM is essential to Munc-13's role in priming (Sudhof and Rizo, 2011). Therefore, it could be that once the vesicle is docked, Munc-13 enables the release of Munc-18 from Syntaxin, allowing it to begin assembly with the other SNARE proteins (Jahn & Fasshauer et al., 2012).

The observation that some clostridial toxins require neural stimulation to exert their destructive effect (Hayashi et al., 1994) led to the hypothesis that the SNARE complex may be partially assembled in a primed state prior to Ca\(^{2+}\) entry and vesicle fusion. Partial assembly of the N termini of SNARE proteins was demonstrated in an elegant experiment by Hua and Charlton (1999), using clostridial toxins which bind to different regions of VAMP. Tetanus toxin and botulinum toxin D bind to the N terminal region of the SNARE motif of VAMP corresponding to layers -5 to -3 while botulinum toxin B binds to a more C terminal location of the SNARE motif between layers +1 and +4. Injection of the catalytic domain of tetanus toxin and botulinum toxin D into a resting neuron caused no effect until the neuron was stimulated. However, proteolysis of VAMP began immediately with the injection of botulinum toxin B. This stimulus dependent effect of the two N-terminally associated toxins indicated that the N terminal region of VAMP may be shielded from attack by partial assembly of the SNARE complex as the vesicles await release in a primed state. The more C terminal region, recognized by botulinum toxin B, however is open to attack, indicating that the complex is not fully assembled prior to Ca\(^{2+}\) evoked fusion (Hua & Charlton, 1999).
Mutations to residues that make up the SNARE protein layers of contact, in a variety of models, were most effective at inhibiting fusion when made in the membrane proximal C-terminal region rather than in the membrane distal N terminal region (Nonet et al., 1998; Fergestad et al., 2001; Sorensen et al., 2006). This supports the hypothesis that the SNARE complex is partially assembled and zips up from N to C termini. In addition, an NMR study of yeast SNAREs concluded that assembly of the SNARE complex proceeds from the membrane distal N-terminal to the membrane proximal C terminal (Fiebig et al., 1999). This concept of progressive assembly of hydrophobic layers of the SNARE complex from N to C termini was thus termed the 'zipper' hypothesis (Sutton et al., 1998). Interestingly, the level of assembly of the primed SNARE complex is still being debated. In a recent review, Jahn and Fasshauer (2012), suggested that prior to Ca$^{2+}$ entry, the SNARE complex may be unassembled.

Now primed and ready for fusion, vesicles are regulated by two important proteins: Synaptotagmin/p65 (Perin et al., 1990) and Complexin (McMahon et al., 1995). As an action potential invades the nerve terminal, it will open voltage gated Ca$^{2+}$ channels, tethered to the active zone by RIM, allowing Ca$^{2+}$ into the neuron (Guy & Conti, 1990). Synaptotagmin/p65, one early identified component of synaptic vesicles (Elferink & Scheller, 1993), contains two Ca$^{2+}$ binding domains and regulates Ca$^{2+}$ evoked vesicle fusion (Brose et al., 1992; Chapman, 2002). Synaptotagmin interacts with phospholipids, Syntaxin, Ca$^{2+}$ channels and other proteins. Importantly, Ca$^{2+}$ has been shown to enhance Synaptotagmin's affinity to the membrane (Brunger, 2000) and Syntaxin (Bennett et al., 1992; Davis et al., 1999; Vrljic et al., 2010). Synaptotagmin may exert its Ca$^{2+}$ dependent role mechanistically through a conformational change observed with Ca$^{2+}$ binding to the protein (Shao et al., 1998). The second critical regulatory molecule is Complexin. Complexin appears to function in a dual role in both promoting and inhibiting fusion. Study of this molecule was difficult because it only binds to the SNARE complex once partial assembly has begun. It resides in a groove on the SNARE complex near the membrane proximal C termini and may block VAMP from joining its partners and completing the SNARE complex (Chen et al., 2002; Lu et al., 2010). Upon Ca$^{2+}$ entry, Synaptotagmin's conformational change may unblock Complexin thus allowing full assembly of the SNARE complex and fusion (Yang et al., 2010). Indeed, deletion of the gene for Complexin results in extremely high levels of spontaneous fusion interpreted as a loss of inhibition (Huntwork & Littleton, 2007). Complexin's role in blocking
fusion at a late stage in priming is hypothesised to be essential to the extreme temporal efficiency of Ca\textsuperscript{2+} evoked transmitter release. While \textit{in vitro} systems require 10 to 100ms after docking for fusion to occur, neurons complete the process in <1ms (Kummel \textit{et al.}, 2011). Recent models predict that Complexin may cross link primed SNARE complexes in a large 'zigzag' array, further stabilizing the pre-fusion state (Kummel \textit{et al.}, 2011).

Once SNARE proteins are allowed to fully assemble, membrane fusion is hypothesized to occur through a series of steps. The first step in membrane fusion is thought to be the formation of a stalk between the outer layer of the vesicle and the inner layer of the plasma membrane (Markin \textit{et al.}, 1984; review by Chernomordik & Kozlov, 2008; Figure 4). This structure was observed in an \textit{in vitro} experiment by creating a stack of two lipid bilayers separated by a layer of water molecules. Upon dehydration, stalk formation readily occurred between the two bilayers (Yang & Huang, 2002). After stalk formation, a hemi-fusion state results where the inner layer of the vesicle comes into contact with the outer layer of the plasma membrane. It has been hypothesized that vesicles may adopt a hemi-fusion state as the last stage of priming before Ca\textsuperscript{2+} triggers fusion (Chernomordik & Kozlov, 2008). Finally, the inner layer of the vesicle combines with the outer layer of the plasma membrane, a pore forms and fusion is complete (Liu \textit{et al.}, 2008). The principle challenges in the fusion of biological membranes is thought to involve forcing the induction of curvature into the bilayers to adopt the various fusion intermediates (Kozlov \textit{et al.}, 2010) and overcoming repulsion from phospholipids and the water molecules that coat the surface of the membrane (Risselada \textit{et al.}, 2011).

While the necessity of SNARE proteins for fusion has been demonstrated many times, the exact number and arrangement of SNARE complexes necessary has been debated. Recent literature has reached somewhat of a consensus with the number of SNARE complexes necessary for neurotransmitter vesicle fusion at 'a few'. Measuring liposome fusion with FRET (fluorescence resonance energy transfer) van den Bogaart \textit{et al.}, (2010), observed fusion which only required single SNARE complexes. A requirement of three SNARE complexes per fusion event was reported by Mohrmann \textit{et al.}, (2010), measured using cultured chromaffin cells. The researchers expressed varying levels of a mutant form of SNAP-25 which did not facilitate vesicle fusion. They observed a decrease in the rate of
vesicle fusion when the mutant version was expressed in combination with the wild-type protein indicating that vesicles required more than one SNARE complex for fusion and that a reduced number of competent SNARE complexes reduced the rate of fusion. By observing the number of fusion events with normal kinetics compared to the overall number of fusion events, the researchers applied a binomial model to estimate that three SNARE complexes are required for normal fusion and suggested that regulation of the number of SNARE complexes per vesicle may be a mechanism to regulate the release probability at a particular type of synapse (Mohrmann et al., 2010). In another study, Sinha et al., (2011), observed VAMP molecules using a pH sensitive fluorophore located at the C terminal, luminal, end of the molecule. The molecule was expressed in hippocampal neurons lacking endogenous VAMP. Upon fusion, the fluorophore moved from the interior of the acidic vesicle to the neutral solution bathing the cells and produced a fluorescent signal. By observing fusion events the authors predicted that two VAMP molecules were the minimal number necessary for fast neurotransmitter vesicle fusion. One final biochemical study estimated the number of SNARE complexes necessary for fusion at one, but that 3 or 4 are required to open a functional pore for diffusion of vesicle contents (Shi et al., 2012). The authors used lipid nano disks with variable numbers of VAMP molecules. They added vesicles with t-SNAREs associated and found that fusion, measured using a fluorescence quenching assay, occurred with as little as one VAMP molecule per nano disk. They also measured exocytosis from the vesicles, which were loaded with Ca^{2+}, using a fluorophore sensitive to Ca^{2+}. They found that 3 or 4 SNARE complexes were the minimal number to achieve maximum exocytosis of cargo from their vesicles. The authors hypothesized that several SNARE complexes are necessary to hold the fusion pore open and that energy released from SNARE complex assembly through the transmembrane domains (Stein et al., 2009) may help force the pore open (Shi et al., 2012). Taken together, these studies point to a minimum requirement of between 2 to 4 SNARE complexes for neurotransmitter vesicle fusion.
Figure 4: Mechanism of fusion

Schematic depicting the mechanism of fusion of the lipid bilayers of the vesicle (blue) and plasma membrane (yellow). The two membranes, originally separate (A) are pulled into close apposition (B). A stalk forms between the outer layer of the vesicle and the inner layer of the plasma membrane (C). Following the stalk, the inner layer of the vesicle comes into contact with the outer layer of the plasma membrane resulting in a hemi-fusion state (D). Finally the fusion pore forms (E) allowing the contents of the vesicle to diffuse into the synaptic cleft. Redrawn from: Markin et al., 1984; Chernomordik & Koslov, 2008.
But does SNARE complex assembly generate enough energy to force membranes together? A recent study by Li et al., (2007) used a surface force apparatus to measure energy associated with SNARE complex formation. Two lipid bilayers were separated by plates with one containing v-SNAREs and one containing t-SNAREs. As the plates were brought into close apposition, association between the SNARE molecules was observed at a distance of 8nm with a sharp adhesion spike. Adhesion continued to rise, albeit more slowly, between 8 and 4nm. The authors concluded that this increase in adhesion represented an increase in energy stored in the SNARE complex. Pulling apart the complex required $35k_BT$ (thermal energy) which the authors describe as energy released during SNARE assembly that would be available for fusion. The energy required for membrane fusion is estimated at 40-50$k_BT$ (Cohen & Melikyan, 2004). Therefore, Li et al., (2007), conclude that $35k_BT$, the amount of energy produced from one SNARE complex, is close to that necessary to initiate fusion.

Studies of viral fusion proteins have concluded that energy released may be applied directly to lipid rearrangements (Durell et al., 1997). Modelling studies have revealed that energy could be transduced through linkers via mechanical coupling (Knecht & Grubmuller, 2003). This release of energy may be used in two different ways to promote fusion. First, energy from SNARE complex assembly may be transduced to transmembrane domains to contribute to membrane bending and stalk formation (Weber et al., 1998). Second, SNARE complex assembly may pull on membrane anchors, dragging associated lipids out of the membrane thus promoting lipid mixing with the closely associated membranes (McNew et al., 2000). These mechanisms may not be mutually exclusive and both could contribute to membrane fusion.

After fusion, vesicle components must be recycled, vesicles endocytosed, refilled with transmitter and transported back to the active zone. The disassembly of the SNARE complex is accomplished by NSF and α-SNAP. NSF binds ATP while the SNARE complex binds 3 α-SNAP molecules. NSF-ATP then binds to α-SNAP, ATP is hydrolysed and the SNARE complex is disassembled (review by Zhao et al., 2012). One proposed mechanism of NSF's role in SNARE complex disassembly is that ATP hydrolysis results in a confirmation change in NSF that generates a mechanical force. This force may be transferred to α-SNAP which binds to grooves in the SNARE complex and may act as lever arms to pry the SNARE
complex apart (Rice & Brunger, 1999). At this point, SNARE proteins can be sorted, vesicles reformed and refilled with neurotransmitter and the cycle repeated again.

Continuing the investigation of SNARE proteins: neuronal-Synaptobrevin/VAMP2

Following the discovery and characterization of SNARE proteins detailed biophysical, biochemical and physiological work by countless workers has begun to reveal the roles and mechanisms performed by this diverse group of proteins. The following work will focus on the neural vesicle associated SNARE: neuronal-Synaptobrevin / VAMP2.

After demonstration of n-Syb's role in neurotransmission in Drosophila through genetic expression of tetanus toxin and creation of a genetic null (Sweeney et al., 1995; Broadie et al., 1995; Deitcher et al., 1998), a similar story began to emerge for the mammal. Knockout mice for VAMP2 die shortly after birth. Synaptic activity measured from cultured hippocampal neurons from these mice show an elimination of evoked neurotransmitter release and reduced, but not eliminated spontaneous neurotransmitter release (Schoch et al., 2001). Further studies of these mice with both VAMP2 and cellubrevin (another VAMP) knocked out, show complete elimination of both evoked and spontaneous release (Borisovska et al., 2005). VAMP is the most numerous protein component of synaptic vesicles with an average of 70 being reported per vesicle in the rat (Takamori et al., 2007).

In Drosophila, two homologous forms of VAMP have been described: Synaptobrevin (syb) and neuronal-Synaptobrevin (n-syb). syb, while expressed most strongly in the gut, is found throughout the organism and likely plays a general role in intracellular trafficking (Sudhof et al., 1989; Chin et al., 1993), while n-syb is found only in neurons (Di Antonio et al., 1993). Flies that are homozygous for a null mutation of n-syb do not develop beyond the embryonic stage and evoked transmitter release is abolished while spontaneous fusion is reduced (Deitcher et al., 1998).
Juxtamembrane region

Proteins of the VAMP family consist of a variable cytosolic N terminal domain, a conserved helix involved in intra-cellular trafficking, a second conserved helix containing the SNARE motif (Figure 3) and a C terminal transmembrane region (TM). Linking the SNARE and transmembrane regions is a short, 10 amino acid, segment called the juxtamembrane region which may also play a role in facilitating membrane fusion (Figure 5a). Figure 5b depicts the juxtamembrane region flanked by the last hydrophobic layer of the SNARE motif (see also Figure 3), leucine 83, and the first hydrophobic residue of the TM domain, methionine 95, which is highly conserved among n-Syb, VAMP2 and other homologs (Elferink et al., 1989; Trimble et al., 1991; Figure 6). Within the juxtamembrane region are several positively charged residues including 5 lysine and one arginine (lysine 82, lysine 84, arginine 85, lysine 86, lysine 91 & lysine 94). In some insects including: Drosophila, Anopheles gambiae (mosquito), Apis mellifera (bee), Nasonia vitripennis (jewel wasp) and Tribolium castaneum (beetle), lysine 91 is replaced by glutamine. The linker domain also has an asparagine residue at position 92. Furthermore, a tryptophan residue is always observed at position 89 and is usually flanked by a tyrosine residue (or phenylalanine in n-Syb) to the amino terminus and another tryptophan residue (or leucine in n-Syb) to the carboxy terminus. A sequence comparison between VAMP2 and n-Syb is depicted in Figure 7. Because the juxtamembrane region of this protein is so well conserved among a diverse group of organisms, a common functional role is likely. Previous studies have implicated this region in several roles including integration of the protein into the membrane after translation, anchoring the protein in the membrane and possible interaction with membrane lipids to facilitate fusion of vesicular and plasma membranes (Durrell et al., 1997; Sutton et al., 1998; Weber et al., 1998; McNew et al., 2000; Tamm & Han, 2000; Van der Wel et al., 2002; Knecht & Grubmuller, 2003).
A.

\[
\begin{align*}
\text{NH}_2 & \quad \text{MGKKDKNKEQADAAPAGDAPPNAGAPAGEGGDGEIVGGPHNPQQIAAQKR} \\
& \quad \text{LQQTQAQVDDEVVDIMRTNVEKVLERDSKLSELDDRADALQQGASQFEQQA} \\
& \quad \text{GKLKRKFWLQNLKMMIIMGVIGLVVVGIIANKLGLIGGEQQPYQPYQPY} \\
& \quad \text{MQPPPPPPPQPPAGGQSSLVDAAGAGDGAGGSSAGAGDHGGV} \quad \text{– COOH}
\end{align*}
\]

B.

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<tr>
<td><strong>D. melanogaster</strong></td>
<td>**NH}_2 85–KRKFWLQNLK–94 COOH (n-Syb)</td>
</tr>
<tr>
<td><strong>R. norvegicus</strong></td>
<td>**NH}_2 85–KRKYWKLQNLK–94 COOH (VAMP2)</td>
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**Figure 5: n-Syb sequence**

A. single letter code amino acid sequence for *D. melanogaster* n-Syb. The amino terminal is cytosolic, while the carboxy terminal is intra-vesicular. Regions are: **SNARE motif** (green), **juxtamembrane region** (blue), **transmembrane region** (bold), **intravesicular region** (purple). B, single letter code amino acid sequence of the juxtamembrane region of n-Syb compared to VAMP2.
**Figure 6: Partial sequence comparison of VAMPs.**

Sequences comparison from the zero layer of the SNARE motif, residue 56 to end or residue 136. SNARE motif, juxtamembrane region (JMR), transmembrane region and intravesicular domain indicated with schematic above. Insect sequences from flybase, others from genbank.
Figure 7: Comparison of *Drosophila* n-Syb and mammalian VAMP2

Full sequence alignment (Clustal W 1.81) of rat VAMP2 and *Drosophila* n-Syb from N- to C- termini. **SNARE motif** (green), **juxtamembrane region** (blue), **transmembrane region** (bold), **intravesicular region** (purple). Note homology in SNARE motif, juxtamembrane region and transmembrane region. *Drosophila* n-Syb contains an extensive intravesicular region that VAMP2 does not.
Integration and trafficking

One well established role for the juxtamembrane region of n-Syb is in functioning as an endoplasmic reticulum (ER) targeting domain. As a Type II protein, the amino terminal of n-Syb is oriented into the cytosol and the carboxy terminal is oriented into the lumen (Trimble et al., 1988, VAMP1). Because proteins are synthesized from N to C termini, n-Syb is not translated directly into the ER membrane, but rather inserted post-translationally. Typically, integral membrane proteins are translated or post-translationally inserted into the membrane via a complex composed of the several proteins that form a channel termed the translocon. Associated with this complex is a signal recognition particle (SRP) bound to an SRP receptor which regulates translation and translocation of the protein through the membrane (review by Martinez-Gil et al., 2011). VAMP however does not utilize the SRP/Sec61p pathway like some type II proteins (Kutay et al., 1995). These proteins however typically do require translocator proteins and utilize ATP to traverse the hydrophobic interior of the plasma membrane (Singer et al., 1987; Whitley et al., 1996). It was demonstrated by Kim et al., (1997), that insertion of VAMP1 into the ER membrane is saturable, indicating that a membrane-bound protein receptor is required for membrane integration. Originally it was proposed that the hydrophobic C terminal sequence was sufficient to integrate and anchor this protein into the membrane (Kutay et al., 1995; Whitley et al., 1996). However Kim et al., (1997), proposed that an insertion sequence was necessary to mediate integration. Kim et al., (1999), demonstrated that four lysine residues: lysine 83, lysine 87, lysine 91 & lysine 94 form the membrane binding signal (VAMP2, Rattus norvegicus). The asparagine at position 92 was also found to be important for integration while mutation of one of the two conserved tryptophan residues (tryptophan 89 and tryptophan 90) did not reduce membrane binding. Also, Quetglas et al., (2002), found that VAMP2 with mutations to the conserved tryptophan residues, transfected into PC12 cells, did not affect trafficking to secretory granules. Thus normal integration occurred with tryptophan mutations. Importantly, n-Syb shares these residues with VAMP2 except for the following: lysine 91 in VAMP 2 is glutamine in n-Syb and tryptophan 90 in VAMP2 is leucine in n-Syb (Figure 5). One major difference between n-Syb and VAMP2 is that n-Syb has a large intra-vesicular domain (Figures 5 & 7). However, this should not change the mechanism of integration because Aplysia VAMP1, which also has
a large intra-vesicular domain, was found to integrate post-translationally into the ER membrane (Yamasaki et al., 1994).

After targeting and insertion into the ER, n-Syb must be transported through the secretory pathway via the Golgi apparatus to synaptic vesicles. A targeting sequence in the cytoplasmic domain of VAMP2 was proposed by Grote et al., (1995). Through deletion mutants they narrowed the putative targeting domain to the first conserved helix comprising residues 41 to 50 (41-EVVDIMRVNV-50). Next, they found that mutating individual hydrophobic residues to alanine (valine 42, valine 43, isoleucine 45, methionine 46, valine 48 and valine 50) blocked targeting. Interestingly they found that mutation of aspartic acid 44 or asparagine 49 to alanine actually increased targeting of the protein to vesicles. The authors suggested that this sequence may interact with other proteins targeted specifically to vesicles and changes observed may result from weaker or stronger binding to these other proteins. The sequence for n-Syb is conserved (41-EVVDIMRTNV-50) potentially indicating a similar function to that observed by Grote et al., (1995). Kutay et al., (1995) proposed that tail-anchored proteins that do not leave the ER use only a hydrophobic signal while those that are transported to other cellular compartments may interact with a membrane receptor protein which then may target the protein to its ultimate destination.

### Role of tryptophan in membrane proteins

Tryptophan residues are common in transmembrane proteins, occurring at high frequency near the ends of transmembrane regions and may play multiple roles including protein insertion into membranes, anchoring and fusion of membranes (Wimley & White, 1996). Tryptophan residues are well conserved in the juxtamembrane region of VAMPs (Weimbs et al., 1998).

Tryptophan and tyrosine residues are thought to determine the precise location of proteins in membranes (Ridder et al., 2000) and have been found to preferentially associate with the membrane-water interface (Yau et al., 1998; Killian & von Heijne, 2000). They may be important in the longitudinal positioning of membrane proteins (Killian & von Heijne, 2000). Ultimately, the preference for tryptophan at interfacial regions may be due to the large
aromatic structure and indole group allowing both hydrophobic and hydrophilic interactions respectively. The rigid aromatic ring from this residue can associate with hydrophobic regions, while the amide group can form hydrogen bonds with the phosphate groups in the membrane (Killian & von Heijne, 2000; see Figure 8). Tryptophan has been found to associate with glycerol groups in phosphatidylcholine membranes (Esbjörner et al., 2007). A recent study using NMR spectroscopy of peptide fragments of the small membrane insertion protein PMP1 (13-GLACIAIIATIYRKWQRQRGLQRF-38) in DPC micelles, found that tryptophan residues were located below but close to the polar head groups of plasma membrane phospholipids (Coic et al., 2005; see Figure 8). Interestingly, this anchoring was not affected by removal of the nearby tyrosine residue. Lysine and arginine may also play a role in membrane anchoring by interacting with both polar and non polar regions of the plasma membrane in a fashion termed ‘snorkeling’ (Killian & von Heijne, 2000). Their long aliphatic side chains preferentially associate with the hydrophobic interior of the membrane while the positively charged amino (lysine) or guanidinium (arginine) head groups may interact with the negatively charged phospholipid head groups or the aqueous solution (Figure 8). Therefore tryptophan and lysine residues in the juxtamembrane region of n-Syb are likely important in anchoring and stabilizing the protein in the membrane.
B.

Indole Ring

Tryptophan

Tyrosine

Phenylalanine

Arginine

Lysine
**Figure 8: Phospholipids and amino acids**

Schematic of the atomic structure of a phospholipid (A) and selected amino acids (B). Phosphatidylethanolamine, the principle component of *Drosophila* membranes, is depicted. The polar head region consists of the ethanolamine group, phosphate group and glycerol group and interacts with hydrophilic species such as water, polar amino acids and each other. The polar head groups arrange in a bilayer protecting the fatty acid tail composed hydrophobic core, from the aqueous solvent. Note, the phospholipid depicted here is abbreviated with only 14 carbon atoms in each fatty acid chain. Amino acids depicted in B, include aromatics tryptophan, tyrosine and phenylalanine and positively charged residues arginine and lysine. Tryptophan typically associates at the membrane interface with the large hydrophobic surface of the indole group allowing association with hydrophobic regions of the membrane and the amide group allowing hydrogen bonding with the phosphate group of the phospholipids. Tyrosine may play a somewhat similar role with a hydrophobic ring structure and a hydroxyl group while phenylalanine is generally hydrophobic in nature. Lysine and arginine are particularly interesting with long aliphatic chains connected to positively charged amino and guanidinium groups respectively. This allows the residues a dual role in associating in the membrane's hydrophobic core while extending their hydrophilic termini to interact with hydrophilic portions of the phospholipid or aqueous media in a fashion termed snorkeling.
Conserved tryptophan residues in the transmembrane region of hemagglutinin, an influenza viral fusion protein, may be also be involved in membrane anchoring (Tatulian & Tamm, 2000). Furthermore, de Haro et al., (2004) found that VAMP constructs with most of the transmembrane region truncated were able to bind to membranes with a physiologically relevant composition of phospholipids (25% phosphatidylserine: 75% phosphatidylcholine). This binding was eliminated when tryptophan 89 and tryptophan 90 were both mutated to alanine. Further evidence for interaction of an interfacial tryptophan with phosphatidylserine in securing a membrane spanning protein was found through mutational analysis of PMP1 (Mousson et al., 2001; 2002). These data show that tryptophan may interact with acidic phospholipids in anchoring the protein in the membrane. A recent liposome binding study found no difference in membrane fusion between liposomes with or without phosphatidylserine (Liu et al., 2008). Besides anchoring, tryptophan residues may be important in stabilizing transmembrane helicies thus maintaining their structure in the membrane bilayer (Ridder et al., 2000). The Ebola fusion peptide (EBO16) with the membrane associated tryptophan residue mutated was found to lose transmembrane helical structure, observed using circular dichrosim spectroscopy, when associated with SDS micelles (Freitas et al., 2007). Therefore this residue in n-Syb may be important in maintaining a rigid transmembrane helix.

In addition to structural roles, the conserved tryptophan residues may be involved in membrane fusion. Tryptophan, strongly anchored to the membrane, may promote lipid mixing as SNARE complex assembly imposes strain on membrane proximal regions of the molecule. Free energy, released during this process, may promote destabilization of the lipid bilayer and thus fusion (Sutton et al., 1998). Force produced through SNARE complex assembly may pull on tryptophan which, if anchored strongly to the membrane, may drag lipids somewhat out of the membrane thereby promoting lipid mixing with the cell's plasma membrane (McNew et al., 2000). Conflicting results have been found from several studies where the tryptophan residues have been mutated. Quetglas et al., 2002, found that by transfecting PC12 cells with VAMP2 constructs, mutated to be immune to tetanus toxin, were able to rescue tetanus toxin inhibited secretion. However, mutation of tryptophan 89 and tryptophan 90 to alanine only restored 25% of release. This supports a role for tryptophan in secretion, but they attributed the effect to an inability to bind Ca^{2+}/calmodulin. They found that the
juxtamembrane region of wild-type VAMP2 was able to bind Ca\textsuperscript{2+}/calmodulin (residues 77-94), with tryptophan 89 and tryptophan 90 being critical (Quetglas et al., 2000). However, other studies have found no evidence for Ca\textsuperscript{2+}/calmodulin regulation of liposome fusion (Siddiqui et al., 2007).

Further data on the role of this region in vesicle fusion was gained from Kweon et al., (2003a) who created tryptophan 89 and tryptophan 90 to serine VAMP2 mutants that were reconstituted into liposomes. The authors proposed that the juxtamembrane region is normally buried in the membrane and changing the tryptophan residues to serine allowed this region of the molecule to move out of the membrane where it could bind with other SNARE molecules. This study supported previously mentioned data showing that the juxtamembrane region is closely associated with the membrane and demonstrated that tryptophan residues are buried beneath its surface. This is contrasted by work from Siddiqui et al., (2007), who found no effect on liposome fusion with tryptophan 89 and tryptophan 90 to alanine mutations. These researchers support the idea that the conserved tryptophan residues are involved in anchoring and stabilizing the protein in the membrane. More recent work using cellular models of fusion has clearly demonstrated an effect of mutation of tryptophan 89 and tryptophan 90 to alanine on fusion. Maximov et al., (2009), found a decrease in the amplitude of evoked potentials and an increase in spontaneous fusion from cultured mouse pyramidal neurons and Borisovska et al., (2012), observed inhibition of the exocytotic burst phase resulting from reduced vesicle priming in cultured chromaffin cells. Therefore, membrane proximal tryptophan appears to play a role in membrane fusion, but is this a result of strong anchoring to the plasma membrane? A combined biophysical and physiological approach to this question may shed light on this debate.

Role of n-Syb/VAMP in membrane fusion

Insight into how biological membranes fuse has been gained from the study of viral and non-viral fusion peptides. Fusion peptides insert and anchor into the host cells membrane and upon a conformational change, the two opposing membranes are brought together. Fusion peptides tend to adopt an angle when passing through a membrane which may promote fusion
SIV fusion peptides (Simian Immunodeficiency Virus; GVFVLGFLGFLA), mutated to reduce angle of insertion (GVFGVALLFLGF), were found to no longer promote fusion. The authors suggested that peptides traversing membranes at an angle may destabilize the membrane by disrupting lipid packing more at the centre than the periphery and induce a negative curvature to the membrane (Epand et al., 1994). In addition, angle of insertion has been correlated with fusogenic activity (Tamm and Han, 2000). VAMP2 has been shown to traverse the membrane at an angle. Initially, Kweon et al., (2003a), found that the first two turns of the membrane helix (residues 85 to 90) show a 33 degree tilt, while Bowen and Brunger, (2006), show that VAMP2 passes through the membrane at angle of approximately 36 degrees to the membrane normal. A recent study of artificial polypeptides with double tryptophan anchors and leucine-alanine-alanine-alanine membrane insertion sequences found that increasing the length, and thus angle, of the transmembrane region lowers the temperature at which membranes will fuse together (Siegel et al., 2006). Similar molecules also integrate into the membrane with a small tilt that is independent of the lipid components or the thickness of the bilayer and the angle may result from tryptophan residues anchoring the peptide (van der Wel et al., 2002). Thus, this angle, implicated to play a functional role in fusion, may be adopted by the fusion peptide from both its length and the structure of the membrane anchor.

Energy transduced to transmembrane regions of fusion peptides could be utilized for fusion in the following ways: pushing against the acyl chains of phospholipids, pushing apart the phospholipid head groups, or dehydrating the surface of opposing membranes. Dehydration, causing a reduction of repulsive force between the two membranes, would allow opposing lipid molecules to interact and mix (Tamm & Han, 2000). The juxtamembrane region of n-Syb could participate in this process because the tryptophan and basic residues integrate into the membrane (Kweon et al., 2003b; Figure 8). Membrane fusion begins with the inner layer of the cell's presynaptic membrane fusing with the outer layer of the vesicle's membrane in a process termed 'hemi-fusion' (Liu et al., 2008). Now, as the SNARE complex completes assembly from the trans to the cis conformation, transmembrane regions from both Syntaxin and VAMP align in the same membrane and fusion is complete (Tamm & Han, 2000).
It has been proposed that the juxtamembrane region may also play a role in the transduction of energy from the formation of the SNARE complex to the transmembrane region thus facilitating fusion (Sutton et al., 1998; Sørensen et al., 2006). The zipper hypothesis suggests that as the SNARE complex forms from N to C termini (Pobbati et al., 2007), energy is released which may be used for membrane fusion. Measurement of the force of assembly of SNARE proteins in an in vitro preparation found an increasing amount of energy stored in the SNARE complex as the artificial membranes were brought closer together. This buildup of energy reached a plateau at a 4nm separation between the two membranes with the C terminus remaining partially unstructured (Li et al., 2007). These results agree with the ‘zipper’ hypothesis and suggest a role for accessory molecules which may prevent full assembly of the SNARE complex until triggered.

Several studies have provided evidence for the ‘zipper’ hypothesis by testing the effect of inserting flexible linkers between the SNARE and transmembrane region on vesicle fusion. McNew et al., 1999, introduced progressively longer glycine-glycine-serine repeats between leucine 93 and lysine 94 and measured fusion of SNARE protein laden liposomes. Using the following constructs: (insert in bold) \(83\text{-KLKRKYWWKLN(KLGGSGGSGGS)13KLKMM-96}\), they found a reduction in fusion with increasing length of the flexible linker (predicted to be helical). The researchers also introduced a pair of proline residues between leucine 93 and lysine 94 and found no effect on fusion. Because proline residues are thought to disturb the alpha helix, they suggested that the reduction of fusion observed in their linker constructs was a result of an increased length rather than increased flexibility. Deak et al., (2006) tested the effect of increased length between the SNARE and linker domain of VAMP2 in rescue experiments of cultured mouse pyramidal cells null for VAMP2 and cellubrevin. They introduced flexible linkers between arginine 86 and lysine 87 (bold): \(83\text{-KLKR(AGS)4KYWWKNLKMM-96}\) and \(83\text{-KLKR(AGS)8KYWWKNLKMM-96}\). While both the 12 and 24 residue linkers were unable to rescue evoked release in the null mutant, the 12, but not the 24 residue linker, did rescue spontaneous release. Interestingly, the mutants and the null showed impaired endocytosis, which was rescued with their wild-type construct. In another report, Kesavan et al., (2007), showed that introducing a flexible linker into the juxtamembrane region had a negative effect on exocytosis from chromaffin cells. Their constructs were similar to those by McNew et al., (1999) where glycine-glycine-serine repeats
were inserted between the juxtamembrane region and transmembrane region. They found an increase in delay to fusion with increasing length of insert, but with no effect on the Ca\(^{2+}\) dependence of release. Ultimately, the burst phase of exocytosis was eliminated with linkers as short as 8 amino acids. A recent report by Borisovska et al., (2012), reported a decrease in the burst phase of secretion from cultured chromaffin cells with a 6 residue flexible insert between leucine 93 and lysine 94. They also tested another insert in the same region which contained tryptophan anchors: (WWKNLK). This allowed a test of increasing length without compromising membrane anchoring. Indeed, a similar decrease in the burst phase was revealed suggesting that increasing length to the juxtamembrane region is deleterious to neurotransmitter vesicle secretion. These studies demonstrate the requirement of a short distance between the SNARE and transmembrane region of VAMP2 for vesicle fusion. They point to an effect of length rather than increased flexibility on the impairment to fusion, however this has not yet been shown with a stiff linker and flexible linker of a given length. A stiff linker could be constructed from helical forming residues such as alanine-alanine-glutamine repeats or using proline residues to form a rigid structure.

Summary

In summary, the juxtamembrane region of \(n\text{-syb}\) has been implicated in several roles in the overall function of the protein. Positively charged amino acids (Figure 5) have been shown to be critical for correct targeting and integration of the protein into the ER membrane (Kim et al., 1999). Furthermore, aromatic residues have been implicated in anchoring and fusion because they orient at interfacial regions and may disturb membrane lipids during SNARE complex formation. Experiments mutating these residues in liposome models have shown no change or an increase in fusion while those conducted in cellular models have shown a marked decrease in secretion. Another potential role for the juxtamembrane region is simply as a short mechanical linker between the SNARE complex and vesicle membrane where membranes could be pulled close together and energy transferred to facilitate membrane bending or lipid mixing. Studies introducing flexible linkers show that increasing length inhibits fusion, but do not lend evidence to support the necessity of a rigid structure.
Therefore there are some conflicting data and open questions regarding the role of the VAMP juxtamembrane region to its role in neurotransmitter vesicle fusion. These disparities may arise because different models have been used to test these ideas, each with strengths and limitations. The liposome model allows fusion to be tested with the minimal compliment of SNARE proteins allowing direct interpretation of changes in protein structure to changes in fusion. But fusion in this model is slow (tens of minutes) while Ca\textsuperscript{2+} activated vesicle fusion in a synapse occurs in a fraction of a millisecond and requires the coordinated interaction of many proteins. Cellular models of fusion such as PC12 and chromaffin cells would measure secretion rather than synaptic transmission. Thus neuronal models, such as cultured hippocampal cells or the \textit{in vivo Drosophila} neuromuscular preparation would be ideal, but with the inverse tradeoff of including all presynaptic structures which may ultimately complicate interpretation of data. However, both resolution of inconsistencies between data from different preparations and detailed analysis of subtle changes to \textit{n-syb} structure to function could be resolved with the \textit{in vivo} synaptic preparation in conjunction with detailed biophysical analysis and the employment of a cellular model.

**Objectives**

Testing the effect of the juxtamembrane region of \textit{n-Syb} on membrane anchoring and neurotransmitter vesicle fusion was conducted using \textit{in vitro} expression in a membrane model and transgenic expression of engineered mutant \textit{n-syb} in \textit{Drosophila}.

The hypothesis that the conserved tryptophan residue in \textit{n-syb} is important for neurotransmitter vesicle fusion requires that it interacts with the membrane. Therefore it was hypothesized that the conserved tryptophan residue in \textit{n-Syb} is important for anchoring and positioning \textit{n-Syb} in the membrane. This hypothesis was tested using \textit{19F} NMR analysis (Chapter 2; Appendix 1). Abbreviated peptides (68 amino acids) including a portion of the SNARE motif, the entire juxtamembrane region, the transmembrane region and a portion of the intravesicular region were produced (Figure 9). A wild-type and two mutants versions with one additional tryptophan residue or with tryptophan mutated to alanine were synthesized with \textit{19F}-labeled aromatic residues and purified into DPC micelles. First, \textit{1H-15N} HSQC spectra were produced to determine if the mutations imposed any significant changes
to secondary structure of the molecules. Next $^{19}$F NMR spectra were collected as a function of temperature to observe the dynamics of the peptides in the DPC micelle. Depth of insertion was first determined by measuring the oxygen induced contact shift. Oxygen, a paramagnetic species, forms a concentration gradient in a micelle under pressure with the highest concentration at the center. The relative position of $^{19}$F labeled reporters could thus be determined based on the magnitude of this shift. A second measure of depth of insertion was provided by observing the solvent induced isotope shift with exposure to $^{2}$H$_2$O (deuterium oxide). A greater exchange was expected in regions of a molecule more exposed to the solvent and thus less buried in the micelle. It was hypothesized that mutation of tryptophan to alanine would decrease stability and increase depth of insertion.
## A. Construct Sequence

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-n-Syb</td>
<td>MGSSHHHHHHSSGLVPRGSHM SQFEQQAGKLRKWFWLQNLKMMIMGVGLVVGGIIANKLGLIGEQ</td>
</tr>
<tr>
<td>WW-n-Syb</td>
<td>MGSSHHHHHHSSGLVPRGSHM SQFEQQAGKLRKWFWQNLKMMIMGVGLVVGGIIANKLGLIGEQ</td>
</tr>
<tr>
<td>AA-n-Syb</td>
<td>MGSSHHHHHHSSGLVPRGSHM SQFEQQAGKLRKFANQNLKMMIMGVGLVVGGIIANKLGLIGEQ</td>
</tr>
<tr>
<td>F77A-n-Syb</td>
<td>MGSSHHHHHHSSGLVPRGSHM SQAEQQAGKLRKFWLQNLKMMIMGVGLVVGGIIANKLGLIGEQ</td>
</tr>
<tr>
<td>Endo-n-Syb</td>
<td>MGKKDKNKEQAADAPAGDAAPPNAGAPAGEEGGDGEIVGGPHNPQQIARQ KLQQTQAQFVDDIMRTNVEKVRSDSKESELDDLADALQQQA SQFEQQAGKLRKWFWLQNLKMMIMGVGLVVGGIIANKLGLIGEQ PPYYYPQQYMQPPPPPQPPQPPAGGQQSSLVDAAGDGAGGGSAGAGDHGGV</td>
</tr>
</tbody>
</table>

## B. WT-n-Syb  C. WW-n-Syb  D. AA-n-Syb  E. F77A-n-Syb
F. Endogenous n-Syb

Figure 9: Constructs for $^{19}$F NMR analysis of n-Syb

A, constructs and endogenous n-Syb. Regions are: *Poly-His and thrombin cleavage* (italics), **SNARE** (green), linker (blue), **transmembrane** (bold), intravesicular (purple), mutations (red), $^{19}$F biosynthetically labelled amino acids (underlined). B, WT-n-Syb showing juxtamembrane tryptophan residue; C, WW-n-Syb showing leucine 90 to tryptophan mutation; D, AA-n-Syb showing tryptophan 89 and leucine 90 to alanine mutation; E, F77A-n-Syb showing phenylalanine 77 to alanine mutation; F, endogenous n-Syb. Note, molecular weight estimated from Expasy bioinformatics resource portal, web.expasy.org. Transmembrane regions estimated with: "Prediction of transmembrane helices and topology of proteins, version 2.0, GE Tusnady", www.enzim.hu/hmmtop/. PDB files modeled using "ESyPred3D", a homology model (Lambert *et al.*, 2002), then displayed using RasMol. Note: molecules are truncated because the known template used to generate PDB files did not have an intravesicular tail (2KOG, Ellena *et al.*, 2009, *R. Norvegicus*, VAMP2). Numbering for n-Syb adapted from VAMP2.
Next, the hypotheses that the conserved tryptophan residue in the juxtamembrane region of n-Syb plays a functional role in neurotransmitter vesicle fusion and that a short juxtamembrane domain provides a mechanical linker to membrane fusion was tested by transgenic expression of engineered mutants in the *Drosophila* model (see Figure 27). *Drosophila* as an animal model offers extensive genetic tools and the well characterized larval neuromuscular preparation for electrophysiological investigation. First, P-element genomic constructs including wild-type, addition of tryptophan, mutation of tryptophan to alanine, insertion of a short, 6 amino acid flexible linker and insertion of a long, 24 amino acid linker, were tested for expression in *Drosophila* S2 cells. Correct expression and membrane integration enabled the production of transgenic flies via P-element transformation.

Transgenic lines produced were characterized for the location of insertion and tested for rescue of an *n-syb* embryonic lethal null mutant and in an *n-syb* hypomorphic background (Chapter 3). The *n-syb* hypomorph, characterized by severe motor deficit and reduced lifespan, was restored to the wild-type condition with transgenic expression of the wild-type *n-syb* transgene thus providing a model with which to test the aforementioned hypotheses. Motor ability of all wild-type and mutant *n-syb* transgenics in the hypomorphic background was tested using a climbing assay and lines selected for further study. Lifespan was also assessed in all transgenic lines in the hypomorphic background.

The potential role for the juxtamembrane region of n-Syb in neurotransmitter vesicle fusion was addressed by testing wild-type and mutant *n-syb* transgenics in the *n-syb* hypomorphic background. Protein expression was measured, development from larval to adult stages was characterized and morphology of the neuromuscular junctions was analyzed. Neurotransmitter vesicle fusion was inferred from sharp electrode intracellular recordings of larval muscles with evoked electrical stimulation of corresponding nerves (0.1Hz). Potential effects of mutations on synaptic plasticity and vesicle trafficking were assessed with higher frequency stimulation (10Hz). Measuring the failure rate with large numbers of stimuli in a low concentration of CaCl₂ was used to determine quantal content. Mutation of tryptophan to alanine was hypothesized to have a detrimental effect on evoked transmitter release while the insertion of an additional tryptophan was not. Insertion of flexible linkers was expected to
have a detrimental effect on evoked transmitter release with increasing length associated with increasing severity.
Chapter 2 Biophysical analysis of the effect of juxtamembrane tryptophan residues using $^{19}$F NMR analysis†

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Author Contributions: Experiments were initiated by C DeMill. Construct design and protein production was performed by C DeMill with assistance from M Serwin. NMR experiments were designed by MS Al-Abdul-Wahid and RS Prosser and conducted by MS Al-Abdul-Wahid. All authors collaborated to guide all aspects of this project including protein production, NMR analysis and manuscript production.

† This chapter has been previously published: Al-Abdul-Wahid MS, Demill CM, Serwin MB, Prosser RS, Stewart BA (2012). Effect of juxtamembrane tryptophans on the immersion depth of Synaptobrevin, an integral vesicle membrane protein. Biochimica et Biophysica Acta (BBA) Biomembranes;1818(12):2994-9.
Abbreviations

wt-TM-n-Syb, residues 75–121 of Drosophila melanogaster n-Synaptobrevin, biosynthetically labeled with 5-fluorotryptophan and 4-fluorophenylalanine; AA-TM-n-Syb, tryptophan 89 and leucine 90 mutated to alanine from wt-TM-n-Syb; WW-TM-n-Syb, leucine 90 mutated to tryptophan from wt-TM-n-Syb

Abstract

Proper positioning of membrane proteins in the host membrane is often critical to successful protein function. While hydrophobic considerations play a dominant role in determining the topology of a protein in the membrane, amphiphilic residues, such as tryptophan, may ‘anchor’ the protein near the water–membrane interface. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family of membrane proteins mediates intracellular membrane fusion. Correct positioning of the SNAREs is necessary if fusion is to occur. Synaptobrevins are integral vesicle membrane proteins that are well conserved across species. Interestingly, mammalian Synaptobrevins typically contain two adjacent tryptophans near the water-membrane interface whereas the Drosophila, neuronal-Synaptobrevin (n-Syb), contains a single tryptophan in this same region. To explore the role of these tryptophan residues in membrane positioning, we prepared a peptide containing residues 75–121 of D. melanogaster n-Syb in DPC micelles, biosynthetically labeled with 4-fluorophenylalanine and 5-fluorotryptophan for the examination by $^{19}$F NMR spectroscopy. Mutations of this construct containing zero and two tryptophan residues near the water-membrane interface resulted in changes in the positioning of n-Syb in the micelle. Moreover, the addition of a second tryptophan appears to slow dynamic motions of n-Syb near the micelle–water interface. These data therefore indicate that juxtamembrane tryptophan residues are important determinants of the position of Synaptobrevin in the membrane.
Introduction

The fusion of cellular membranes, guided by key proteins that mediate membrane disruption and association, presents a fundamental biophysical problem where the repulsive forces between membranes must be overcome (Chernomordic & Kozlov, 2003). A solution to this problem is achieved with the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) super family of membrane proteins (Brunger 2005; Jahn & Scheller, 2006). These proteins share an evolutionally conserved motif of 60–70 amino acids (Sutton et al., 1998), and are located in both the vesicle and target membranes. During membrane fusion, SNAREs from both target and vesicle membranes combine to form a four-helix complex. A well-characterized example of SNARE mediated fusion is neuronal exocytosis, where two cellular membrane proteins, syntaxin-1A (Bennett et al., 1992), and SNAP-25 (synaptosomal associated protein of 25 kD) (Oyler et al., 1989) complex with VAMP-1/2 (vesicle associated membrane protein, also referred to as Synaptobrevin) (Trimble et al., 1988; Elferink et al., 1989). It is thought that the complex remains partially assembled (Hua & Charlton, 1999), until calcium binding to syntaptotagmin (Pang et al., 2006) triggers full assembly. Energy released (Sutton et al., 1998) upon formation of the ternary stable parallel coiled-coil (Poirier et al., 1998) SNARE complex is believed to provide the energy necessary for membrane fusion (Lin & Scheller, 1997; McNew et al., 1999; McNew et al., 2000).

Of particular interest is the VAMP subfamily of SNARE proteins, which have been characterized across a wide range of species, including yeast (Gerst et al., 1992), Drosophila melanogaster (Sudhof et al., 1989), rat (Baumert et al., 1989; Trimble et al., 1990), Torpedo californica (Trimble et al., 1988), and human (Archer et al., 1990). VAMPs consist of a variable N terminal domain, a conserved SNARE motif, a conserved linker (or juxtamembrane) region, a conserved transmembrane region and a variable intravesicular C terminal region. While much attention has been focused on studies of the SNARE motif, less has been paid to the linker region which contains highly conserved aromatic and positively charged residues. These residues have been shown to interact with membrane phospholipids (Esbjorner et al., 2007; Killian & von Heijne, 2000) and may be important for membrane fusion. Moreover, it has been proposed that these interactions play a role in lipid mixing
during SNARE complex formation (Sutton et al., 1998; Kweon et al., 2003a). Additionally, several of these residues may interact with Syntaxin during the final stage of SNARE complex formation and membrane fusion (Stein et al., 2009). Thus, aromatic and charged residues in the linker region may be essential for membrane fusion.

The linker region of mammalian VAMP2 contains two consecutive tryptophan residues (positions 89 and 90) which have been reported to localize at the membrane–water interface (Kweon et al., 2003a). Mutation of these residues to alanine severely inhibits neuronal exocytosis in cultured mouse hippocampal neurons with complete loss of evoked transmitter release and yet an increase in spontaneous fusion (Maximov et al., 2009). Other works assessing fusion with mutations in this region have produced conflicting results with no effect on fusion of liposomes (Siddiqui et al., 2007), an increase in fusion of liposomes (Kweon et al., 2003b) and decreased exocytosis from PC12 cells (Quetglas et al., 2002). The mechanisms underlying these divergent results have yet to be fully described.

Due to the amphipathic nature of the membrane–water interface, membrane proteins are often anchored to the membrane by distributing tryptophan and tyrosine residues at the interface (von Heijne, 1994; Yau et al., 1998; Landolt-Marticorena et al., 1993). In fact, the position of tryptophan residues in a membrane protein can have profound effects on immersion depth and protein orientation (de Planque et al., 2003). Tryptophan residues have been suggested to serve as “anchors” at the membrane interface (Ridder et al., 2000; Kachel et al., 1995; Laco & Pommier, 2008), thus stabilizing transmembrane regions of the protein. This may allow force, generated during SNARE complex assembly, to be transduced to the membrane (Sutton et al., 1998; Hu et al., 2002; Jahn & Sudhof, 1999). Indeed, de Haro et al., found that mutation of tryptophan residues increased water exposure of this normally buried region of the molecule (de Haro et al., 2004). In VAMP2, strong anchoring by tryptophan (residue 89), along with presence of a lysine (residue 94) just inside the membrane, may force the transmembrane region to adopt an oblique 36° angle with respect to the membrane which is thought to promote fusion (Bowen & Brunger, 2006).

Tryptophan’s fundamental role in membrane fusion is exemplified through work on viral fusion proteins. Mutation of tryptophan and other aromatic residues in herpes simplex virus fusion proteins reduced both membrane association and fusogenic activity (Galdiero et
Similarly, mutation of aromatics inhibited membrane fusion of HIV (Salzwedel et al., 1999), and influenza (Lai & Tamm, 2007). In addition, tryptophan has been shown to be important in membrane anchoring and maintenance of secondary structure in Ebola fusion proteins (Freitas et al., 2007). Interestingly the biophysical challenges that need to be overcome and mechanisms employed are similar for membrane fusion by both viruses and cells. This includes strong anchoring of proteins to membranes that are subsequently forced into close apposition causing first hemi-fusion and then full fusion (see White et al., 2008 for a review). Therefore, studying the role of tryptophan in membrane anchoring and fusion across various biological models provides insight fundamental to biology.

The VAMP family member responsible for neurotransmitter release in *Drosophila melanogaster* is known as neuronal-Synaptobrevin (n-Syb) (Deitcher et al., 1998), and it provides a unique tool to study this mechanism because it possesses only one tryptophan residue (residue 89) in the linker region (DiAntonio et al., 1993). Does the presence of a second tryptophan significantly improve anchoring of the protein in the membrane? Conversely, how does the substitution of tryptophan with another residue affect n-Syb topology? In this work we explore the effect of tryptophan residues on the immersion depth of n-Syb peptides in DPC micelles, using constructs that contain zero (tryptophan 89 and leucine 90 mutated to alanine), one (wild-type), or two (leucine 90 mutated to tryptophan) tryptophan residues.

**Observing n-Syb by $^{19}$F NMR**

The $^1$H nuclei naturally present in proteins are readily detected by NMR, and for small membrane peptides where the $^1$H spectra are well-resolved, immersion depth studies require no modification or isotopic enrichment of the protein (Respondek et al., 2007). The $^1$H spectrum of n-Syb, however, contains significant overlap of peaks and is too complex to be assigned. In such cases, NMR studies typically rely on one of the two methods to improve spectral resolution: isotopic enrichment or the incorporation of an NMR-active probe moiety. Isotopic enrichment, with $^{15}$N and $^{13}$C, allows multidimensional NMR experiments and the concomitant increase in spectral resolution. Alternatively, the introduction of specific NMR probes, via biosynthetic labeling or chemical modification, provides a means of studying
specific sites on the protein, reducing extraneous information and obviating the time-consuming assignment of $^{15}$N, $^{13}$C-labeled proteins. Moreover, large proteins labelled may be studied via one-dimensional NMR spectroscopy, requiring less spectrometer time than a multidimensional NMR-based approach. In this work, 5-fluorotryptophan and 4-fluorophenylalanine are introduced biosynthetically into n-Syb and its two mutants, providing two to four $^{19}$F reporter groups. This simple substitution, of a $^1$H atom for a $^{19}$F atom, provides a powerful tool to study n-Syb, owing to the high gyromagnetic ratio (94% that of $^1$H), absence of $^{19}$F in natural systems, and the high sensitivity of the $^{19}$F chemical shift to changes in local environment. In particular, phenylalanine 88, which is adjacent to the anchoring tryptophan residue in n-Syb, reports directly on the immersion depth of residues in this region.

Materials and Methods

The cDNA clone for n-Syb in a pOT2 vector (isoform PE; GH4664; chloramphenicol resistance) was obtained from the Drosophila Genomics Resource Center. Primers (Sigma-Aldrich) incorporating NdeI and BamHI restriction sites were used to PCR amplify the coding sequence for the 47 residue peptides (Residues 75–121, SQFEQQAGKLKRKFWLQNLKMMIIMGVIGLVVVGIIANKLGLIGGEQ) encoding a portion of the SNARE motif, the entire linker and transmembrane regions and a portion of the intravesicular domain. The PCR product was purified in 3.5% low melting temperature gel (NuSieve GTG Agarose, Mandel). pET15b vector (Novagen; ampicillin resistance) was digested with NdeI and BamHI, CIP treated and gel purified. The vector was combined with the PCR product, ligated at room temperature overnight and transformed into DH5α competent cells (New England Biolabs). Site directed mutagenesis was employed to produce the following clones: leucine 90 mutated to tryptophan (WW-TM-n-Syb), tryptophan 89 and leucine 90 mutated to alanine (AA-TM-n-Syb), and phenylalanine 77 mutated to alanine, which were subsequently confirmed by sequencing (ACGT Corp, Toronto). Clones were transformed into BL21 (DE3) competent cells (Novagen) along with a vector (Magic, kanamycin resistance) incorporating coding for rare tRNA (gift from Dr. W.S. Trimble, University of Toronto). The Magic plasmid encodes three tRNA molecules which recognize
the following nucleotide sequences: adenine-guanine-guanine, (arginine); adenine-guanine adenine (arginine); adenine-thymine-adenine (isoleucine) (Wu et al., 2000). The final 68 residue peptide incorporated a 6xHis-tag and thrombin cleavage site (MGSSHHHHHSSGL-VPRGSQFEQQAGKLRKFWLQNLKMMIIMGVIGLVVGVGIIANLKGLIGGEQ).

Protein expression in M9 minimal media and subsequent purification was performed based on an established method for membrane proteins (Oxenoid et al., 2004), and is detailed in the supporting information. 5-fluorotryptophan and 4-fluorophenylalanine-labeled samples were produced by glyphosate (N-(phosphonomethyl)glycine) induced auxotrophy of the production of aromatic amino acids (Kitevski-Leblanc et al., 2009). Briefly, as cell cultures reached an OD of 1.0, glyphosate (1 g/L) was added to stop the production of phenylalanine, tryptophan, and tyrosine residues by E. coli. Unlabelled tyrosine (37.5 mg/L) was also added at this time. After waiting for 1 h to ensure depletion of any remaining tryptophan and phenylalanine residues, 5-fluorotryptophan (36 mg/L) and 4-fluorophenylalanine (36 mg/L) were added to the cell culture, and protein expression was immediately induced using IPTG. After 6 h, cells were collected by centrifugation and stored at −20 °C. Protein purification (supporting information) was conducted using a denaturing protocol based on that described by Sanders and co-workers (Tian et al., 2005).

NMR experiments were performed on a 600 MHz Varian Inova spectrometer equipped with a triple resonance cold probe capable of tuning to $^{19}$F (564 MHz), or a triple resonance room temperature probe also capable of tuning to $^{19}$F. Typical HSQC spectra were acquired with 64 scans per increment and 160 increments, and processed with the NMRPipe processing suite (Delaglio et al., 1995). $^{19}$F 1D spectra were typically acquired in 1 h, with 2048 scans and an interscan delay of 1.3 s. $^{19}$F spectra were processed using both VNMRJ (Agilent) and MestReNova (Mestrec). A fifth order polynomial baseline correction was applied to remove $^{19}$F signals arising from the fluorinated materials in the probe assembly.

$O_2$-PRE values were obtained using a 5 mm sapphire NMR tube (Saphikon, NH), with a home-built Swage-lok fitting. Samples were pre-equilibrated at an $O_2$ partial pressure of 20 bar for at least two days prior to measuring relaxation rates to ensure a constant $[O_2]$ during the experiments. Relaxation rates under diamagnetic conditions were acquired on samples pressurized with He gas to a pressure of 20 bar.
Solvent-induced isotope shifts were obtained as the difference in chemical shift for samples in pure (99%) D₂O (Cambridge Isotopes Laboratories, Andover, MA) and samples in 10% D₂O/90% H₂O (i.e. SIIS=δ₁₀₀% D₂O−δ₁₀% D₂O/90% H₂O). Sodium fluoride (1 mM) was added to all samples as a chemical shift reference. In a separate experiment, the precise chemical shifts of NaF in 100% D₂O and in 10% D₂O/90% H₂O were simultaneously measured using a coaxial NMR tube.

Results and Discussion

To confirm that our desired mutations did not alter the overall structure of n-Syb, we collected NH-HSQC spectra of ¹⁵N-labeled samples of each construct. The NH-HSQC is a fast method of ‘fingerprinting’ the backbone conformation of a sample. Peaks produced result from correlation of chemical shifts of directly bound ¹⁵N and ¹H nuclei. Each amino acid, with the exception of proline, will thus produce a characteristic peak from the peptide backbone nitrogen and corresponding hydrogen. Additional peaks are produced from nitrogen containing side groups found in residues such as asparagine, glutamine and tryptophan. If a mutation does not affect the overall structure, the ‘fingerprint’ will be very similar (i.e. only the mutated residues will have large chemical shift changes). On the other hand, any mutations affecting the overall structure of the peptide (e.g. the breakage of a disulfide bond) will cause large changes in the chemical shifts of multiple peaks, resulting in a different ‘fingerprint’. The spectra of the wild-type fragment (wt-TM-n-Syb), the tryptophan 89 and leucine 90 to alanine mutant (AA-TM-n-Syb) and the leucine 90 to tryptophan mutant (WW-TM-n-Syb) are very similar (Figure 10; Figure 42), indicating that the mutations do not affect the overall structure of the protein. ¹⁹F spectra of wt-TM-n-Syb (Figure 11) exhibit three peaks: one from the 5-fluorotryptophan at residue 89 (−124.6 ppm) and two from the 4-fluorophenylalanines at residues 77 (−116.1 ppm) and residue 88 (−115.6 ppm). The two 4-fluorophenylalanine residues were assigned using a phenylalanine 77 to alanine mutant of wt-TM-n-Syb (data not shown).
Figure 10: NH-HSQC spectra of n-Syb wild-type and mutant constructs

wt-TM-n-Syb (left), AA-TM-n-Syb (centre), and WW-TM-n-Syb (right). Mutants produced were: "AA" mutation of tryptophan 89 to alanine and leucine 90 to alanine; "WW" mutation of leucine 90 to tryptophan. Both the "AA" and "WW" mutants exhibit similar HSQC spectra as the wild-type, indicating that the mutations do not grossly alter the structure of n-Syb. See appendix 1, figure 42 for expanded spectra and overlay.
Effects of temperature on the immersion depth of phenylalanine 88

$^{19}$F chemical shifts are highly sensitive to changes in local environment. For $^{19}$F atoms in membrane proteins, an increase in the immersion depth in the micelle/bilayer results in a downfield shift of $^{19}$F resonances (Hull & Sykes, 1976) due to an increase in the number of nearby aliphatic $^1$H atoms. To examine the ability of tryptophan to ‘anchor’ n-Syb in the micelle, we collected $^{19}$F 1D spectra as a function of temperature (Figure 11), and considered the temperature dependence of the $^{19}$F chemical shifts. In order to account for the sensitivity of the $^{19}$F chemical shift to temperature (i.e. in the absence of changes in immersion depth), we also collected $^{19}$F spectra of free 4-fluorophenylalanine in aqueous solution, and observed a small (<0.1 ppm/10 °C) temperature dependence on chemical shift (Figure 16). In wt-TM-n-Syb, both phenylalanine 77 and tryptophan 89 exhibit similar (<0.1 ppm/10 °C) small temperature dependences on their chemical shifts, suggesting no change in environment (Figure 16, Figure 17). Meanwhile, phenylalanine 88 shows a pronounced downfield shift (~0.2 ppm/10 °C) at lower temperatures (Figure 11), indicating that phenylalanine 88 is more deeply embedded in the micelle at lower temperatures. Interestingly, at these colder temperatures, downfield shifts are accompanied by an increase in peak line width of phenylalanine 88. Such increases are consistent with exchange broadening resulting from sampling two different chemical environments.

Taking into account the proximity of phenylalanine 88 to the micelle–water interface, the increase in the peak line width of phenylalanine 88 at lower temperatures suggests that the $^{19}$F atom ‘criss-crosses’ the micelle–water interface (e.g. a ‘bobbing’-type motion) (Figure 12). In n-Syb, the amplitude of the motions leading to this exchange must be small, as the adjacent tryptophan 89 residue is not strongly broadened, nor does tryptophan 89 exhibit downfield shifts at lower temperatures, indicating that tryptophan 89 does not undergo a change in local environment. Therefore, if the motions giving rise to the observed phenylalanine 88 dynamics are in fact ‘whole-body’ motions, the amplitude must be sufficiently small to keep the chemical environment surrounding tryptophan 89 unchanged. More likely, the motions are localized to the phenylalanine 88 side chain.
To determine the effect of adding or removing tryptophan residues on the dynamics of n-Syb in the vicinity of phenylalanine 88, we compare the temperature dependence of the phenylalanine 88 chemical shift in the “AA” and “WW” mutants to that of the wild-type (Figure 13). Replacing tryptophan 89 with an alanine (i.e. AA-TM-n-Syb) does not affect the temperature dependence of the phenylalanine 88 chemical shift; both the line broadening (not shown) and downfield shift of phenylalanine 88 are similar to the wild-type construct. The removal of the juxtamembraneous tryptophan does not appear to significantly alter n-Syb dynamics near the micelle–water interface.

Adding a second tryptophan near phenylalanine 88 (i.e. WW-TM-n-Syb; mutation of leucine 90 to tryptophan) changes the chemical shift profile of phenylalanine 88 (Figure 13). Here, phenylalanine 88 exhibits only small up field shifts at low temperature, consistent with the normal temperature dependence of 19F-bearing residues not undergoing a change in local environment. We conclude that the addition of a second tryptophan ‘anchors’ the position of phenylalanine 88 in the micelle, reducing the motions that give rise to chemical exchange.
Figure 11: $^{19}$F NMR spectra of wt-TM-n-Syb biosynthetically labeled with 5-fluorotryptophan and 4-fluorophenylalanine

Temperatures were 293 K (a), 310 K (b) and 318 K (c).
Figure 12: Cartoon schematic of a $^{19}$F-Phe residue in an alpha helical protein embedded in a detergent micelle (similar to the $^{19}$F-labelled phenylalanine 88 residue of n-Syb).

Translational motion of the $^{19}$F atom, as suggested by the double arrow, would give rise to chemical exchange between water-exposed and micelle-exposed states. Exchange rates on the order of the difference in chemical shift between the two states result in an increase of NMR linewidths, as observed for wt-TM-n-Syb.
Figure 13: $^{19}$F chemical shift of 4-fluorophenylalanine biosynthetically substituted for phenylalanine 88 as a function of sample temperature

wt-TM-n-Syb (blue diamonds), AA-TM-n-Syb (red circles) and WW-TM-n-Syb (green squares).
Oxygen-induced paramagnetic contact shifts of phenylalanine 88

Oxygen is a paramagnetic compound; the unpaired electrons of O\textsubscript{2} alter the chemical shifts of nearby \textsuperscript{19}F nuclei. The magnitude of this phenomenon, termed the contact shift, scales with local [O\textsubscript{2}]. Owing to the polarity gradient across the micelle, local [O\textsubscript{2}] is a function of immersion depth in the micelle; at a partial O\textsubscript{2} pressure of 20 bar and a temperature of 45 °C, local [O\textsubscript{2}] spans a factor of seven, from ~15 mM in the bulk water to ~105 mM in micelle core (Windrem and Planchy, 1980). O\textsubscript{2} contact shifts (Figure 14) thus provide a measure of the relative immersion depth of phenylalanine 88 across the three proteins, with a larger contact shift indicating greater burial in the micelle.

Relative to the wild-type construct, the loss of the tryptophan and introduction of two hydrophobic alanine residues pulls the phenylalanine 88 of AA-TM-n-Syb deeper into the micelle, as evidenced by the larger contact shift (Figure 14). Conversely, for the wild-type construct, W89 preferentially resides near the micelle–water interface, relegating phenylalanine 88 to a more water-exposed region than in AA-TM-n-Syb, where the observed contact shift is smaller. The addition of a second tryptophan in this region marginally reduces the contact shift of phenylalanine 88, suggesting that the average immersion depth of phenylalanine 88 is not substantially affected by the introduction of a second tryptophan, although this does not preclude the reduction of exchange dynamics by a second tryptophan anchor.
Figure 14: Oxygen-induced contact shifts of 4-fluorophenylalanine biosynthetically substituted for phenylalanine 88

Oxygen-induced contact shifts of 4-fluorophenylalanine biosynthetically substituted for phenylalanine 88 for all three n-Syb constructs, arising from an O$_2$ partial pressure of 20 bar, at 45°C. Contact shift values are equal to $\delta$(O$_2$) – $\delta$(He). Error bars indicate the precision with which chemical shifts of F88 could be determined and are approximately half the line width of the $^{19}$F peaks.
Solvent-induced isotope shifts of phenylalanine 88

The sensitivity of $^{19}\text{F}$ chemical shifts to their local environments is such that the substitution of H$_2$O for D$_2$O in the buffer shifts peaks by as much as 0.25 ppm (Gerig, 1994). The magnitudes of these solvent-induced isotope shifts (SIIS) scale with local water concentration and serve as another measure of micelle immersion depth. SIIS values of zero are expected for nuclei located in the micelle center, where water is excluded (Hull & Sykes, 1976; Hagen et al., 1979). At the micelle/water interface, where the [H$_2$O] gradient is steep, SIIS values are a function of the immersion depths of $^{19}\text{F}$ nuclei in this region (Hansen et al., 1985). The measurement of SIIS for the three n-Syb constructs (shown in Figure 15) complements the earlier analysis of O$_2$-contact shifts.

Consistent with the above analysis of the O$_2$-contact shift data, the degree of water exposure at phenylalanine 88 increases with increasing tryptophan content. Comparatively low SIIS values for the phenylalanine 88 residue of AA-TM-n-Syb are consistent with our assertion that the hydrophobic tryptophan 89 and leucine 90 to alanine substitutions drag phenylalanine 88 into the micelle. In wt-TM-n-Syb, the anchoring of tryptophan 89 near the micelle–water interface places phenylalanine 88 in a more water exposed region, as evidenced by the greater magnitude of the phenylalanine 88 SIIS in this construct. The addition of a second tryptophan (i.e. WW-TM-n-Syb) serves to further push phenylalanine 88 away from the micelle. The magnitudes of the SIIS for all three constructs increases with temperature, indicating a preference for the micelle-bound state at lower temperatures.
Figure 15: $^{19}$F solvent induced isotope shifts (SIIS) of 4-fluorophenylalanine biosynthetically substituted for phenylalanine 88

$^{19}$F solvent induced isotope shifts (SIIS) of 4-fluorophenylalanine biosynthetically substituted for phenylalanine 88 of wt-TM-n-Syb (blue diamonds), AA-TM-n-Syb (red circles) and WW-TM-n-Syb (green squares) as a function of sample temperature. SIIS values are equal to $\delta$(D$_2$O) - $\delta$(H$_2$O); SIIS values of zero indicate no exposure to water, while increasingly negative values suggest greater exposure. Samples contained 1 mM sodium fluoride, which was used as a chemical shift reference.
Conclusions

A $^{19}$F NMR analysis reveals the important role of tryptophan residues on membrane anchoring and dynamics of neuronal-Synaptobrevin. Mutation of the interfacial tryptophan residue 89 in n-Syb allows hydrophobic considerations to dictate n-Syb immersion depth, resulting in phenylalanine 88 being pulled deeper into the micelle. The presence of an interfacial tryptophan residue anchors n-Syb such that phenylalanine 88 is raised out of the micelle, into a water-exposed region.

$^{19}$F chemical shifts and line widths suggest that AA-TM-n-Syb and wt-TM-n-Syb undergo some form of chemical exchange at lower temperatures. We envisage this as a “bobbing” type motion; as phenylalanine 88 is expected to reside near the micelle/water interface, local motions of n-Syb may result in phenylalanine 88 bobbing back and forth between two regions with differing degrees of water exposure. We observed the elimination of this chemical exchange upon the addition of a second tryptophan residue (mutation of leucine 90 to tryptophan) near phenylalanine 88, which we attribute to the increased anchoring effect of the two adjacent tryptophan residues.

We know from previous work that proteins of the Synaptobrevin family are critical for vesicle fusion in general, and neural synaptic transmission in particular. For example, the loss of Synaptobrevin, through genetic techniques, or cleavage of the protein by tetanus toxin, profoundly impairs synaptic vesicle release (Schiavo et al., 1992; Sweeney et al., 1995). Aromatic and charged residues are highly conserved in the juxtamembrane region of Synaptobrevins and may play a role in vesicle fusion.

The role of tryptophan as an anchor to precisely position a protein in a membrane has been demonstrated (Esbjorner et al., 2007; Killian & von Heijne, 2000). A functional role for tryptophan has also been shown since elimination of tryptophan residues from viral fusion proteins inhibits viral fusion (Galdiero et al., 2008; Salzwedel et al., 1999; Lai & Tamm, 2007). In a physiological study of VAMP2, the mutation of tryptophan in the linker region to alanine, has been shown to negatively affect neurotransmitter release in cultured hippocampal cells (Maximov et al., 2009), and exocytosis from PC12 cells (Quetglas et al., 2002). It has been hypothesized that strong anchoring of the protein in the membrane by tryptophan is
necessary to efficiently transfer energy during SNARE complex to complete membrane fusion (Lin & Scheller, 1997; McNew et al., 1999; McNew et al., 2000). However, to our knowledge, no atomic level structural studies of tryptophan to alanine mutations in the Synaptobrevin family have been performed to support this hypothesis. Thus, our work on n-Syb clearly demonstrates the role of tryptophan in anchoring and positioning the protein in the membrane and supports the hypothesis that the deleterious physiological affects observed in different systems may result from decreased anchoring.

While highly conserved, the amino acid sequence of the juxtamembrane region of n-Syb differs from VAMP2 (n-Syb:84-KLKRKFWLQNLK-94; VAMP2:84-KLKRKYWWKNLK-94; see Figure 5). The fact that n-Syb has only one tryptophan residue presented us with the opportunity to test the effect of either the addition or removal of one tryptophan on the native structure. The decision to produce a double alanine mutant (i.e. AA-TM-n-Syb) rather than a single tryptophan 89 to alanine mutation was made to allow comparison to previous work on double alanine mutants of VAMP2 (Maximov et al., 2009; Siddiqui et al., 2007; Quetglas et al., 2002). Although mutation of leucine 90 for the less hydrophobic alanine residue may have decreased the immersion depth of phenylalanine 88 in the micelle, we expect this decrease to be minor, without any significant impact on the findings of this study. Interestingly, adding one tryptophan did increase peptide anchoring to the micelle, which is consistent with the anchoring hypothesis. At present we cannot answer the question of why Drosophila n-Syb has only one tryptophan while mammalian VAMP2 has two, because there are other differences between the two proteins that we have not yet accounted for. Our WW-TM-n-Syb construct is simply n-Syb with an additional tryptophan and differs from VAMP2 in the SNARE, linker and transmembrane regions. An interesting area to explore is the relationship of these VAMP/Synaptobrevin family members to other species-specific differences in membrane composition or accessory protein structure. For example, mammalian membranes are high in phosphatidylcholine with a substantial amount of the negatively charged phospholipid phosphatidylserine, while Dipteran membranes are predominantly composed of phosphatidylethanolamine with very little phosphatidylserine (Luukkonen et al., 1973). Answering these questions using different experimental systems, across a variety of model organisms, will help determine the fundamental role that aromatic and charged residues play in membrane fusion.
Altogether our data support the idea that tryptophan residues near the membrane–water interface are important determinants of Synaptobrevin peptide depth within the micelle environment. Our future work will address the physiological importance of these results by introducing these mutants into Drosophila synapses using transgenic technology.

Supplementary Data

Supplementary Methods

Phosphorylated primers (Sigma Aldrich) used for site directed mutagenesis of n-Syb.

L90W, PF:AAGAGGAAATTCTGGTGCCAGAACTTAAG, PR:GAGCTTGCCCCTGCTG; tryptophan 89 and leucine 90 to alanine,

PF:AAGAGGAAATTCCGGCTCAGAACTTAAG, PR: same as previous; F77A, PF: CCATATGTCCAGGCAGAGCAGCAGGCGG, PR:CTGCCGCGCGGCACCAGGCC.

Peptides were produced by first growing single transformants in LB broth with ampicillin (100 mg/mL) and kanamycin (50 mg/mL) for 6 hrs at 37 °C. 40mL of this starter culture was centrifuged (1500 rpm, 10 min, 15 °C) and the resulting pellet used to inoculate 1 L of minimal media. Each litre of minimal media contained (6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g $^{15}$NH₄Cl, 1 mL 0.1 M CaCl₂, 1 mL of 1M MgSO₄, 10 mL of 40% (w/v) glucose, 2 mL of a crushed vitamin extract (Life brand adult multi-vitamins and minerals) and 100 mg ampicillin, adjusted to pH 7.0 as previously described (Oxenoid et al., 2004). The vitamin extract was obtained by crushing the vitamin, adding the powder to 20 mL H₂O, vortexing for 5 minutes and then sonicating for 5 minutes in a bath sonicator. The supernatant was cleared by centrifugation and passed through a sterile filter (Chuck Sanders, personal communication). Typically, 3 L cultures were grown for 8 hrs on a shaker at 37 °C. Upon reaching an OD greater than 1, glyphosate (1 g/L) and tyrosine (37.5 mg/L) were added to the culture (Kitevski-Leblanc et al., 2009). After 1 h, 5-fluorotryptophan (36 mg/L), 4-fluorophenylalanine (36 mg/L) and IPTG (238 mg/L) were added. Cultures were grown for 6 hrs, and then centrifuged (6000 rpm, 20 min, 4 °C). Pellets were stored at -20 °C overnight.
Protein purification was conducted using a denaturing protocol described by Tian et al., (2005). Briefly, cells were resuspended in 20 mL/g of cells in lysis buffer (75 mM tris-HCl, 0.3 M NaCl, 0.2 mM EDTA, 10 μM BHT, pH 7.7) plus PMSF (20mg/100mL), lysozyme (0.2 mg/mL), DNase (0.02 mg/mL) and RNase (0.02 mg/mL). Cell lysis and purification were performed with repeated sonication and centrifugation (12000 rpm, 15 min, 4 °C) where soluble portions were discarded and the pellet was saved. The remaining insoluble pellet was resuspended in SDS-urea (20 mM tris-HCl, 200 mM NaCl, 8 M urea, 0.2% SDS, pH 8). This buffer was centrifuged (15000 rpm, 20 min, 15 °C) and supernatant combined with nickel-nitritotriacetic acid (Ni-NTA) agarose resin (Qiagen). Peptide, now bound to Ni-NTA resin, was purified using gravity-flow chromatography in a Kontes Flex Column (Fisher Scientific). Elution was performed in buffer containing DPC (dodecyl phosphatidylcholine) (5 mM Tris, 50 mM NaCl, 250 mM imidazole, 0.5% DPC, pH 7.5). Peptide concentration and buffer exchange was performed in centrifuge tubes with a 3 KDa cutoff filter (Amicon). Peptide production was confirmed with Coomassie blue staining and Western blot. Concentrations of samples were determined with a Nano Drop spectrophotometer.
Supplementary Results

Figure 16: $^{19}$F chemical shifts of 4-fluorophenylalanine biosynthetically substituted for F77 as a function of sample temperature.

wt-TM-n-Syb (blue diamonds), AA-TM-n-Syb (red circles) and WW-TM-n-Syb (green squares). The chemical shifts of free 4-fluorophenylalanine in aqueous solution are shown for comparison (black triangles).
Figure 17: $^{19}$F chemical shifts of 5-fluorotryptophan biosynthetically substituted for W89 as a function of sample temperature.

wt-TM-n-Syb (blue diamonds) and W89/W90 of WW-TM-n-Syb (green squares). The WW-TM-n-Syb chemical shift is the average of the chemical shifts of tryptophan 89 and tryptophan 90. The chemical shifts of free 4-fluorophenylalanine in aqueous solution are shown for comparison (black triangles), and are indicated on the right y-axis. Both axes are drawn to the same scale.
Chapter 3: Transgenic rescue of n-syb mutants Using a Reverse Genetic Strategy

Introduction

Forward genetic screens have provided tremendous scientific insight into the function of genes and proteins; a reverse genetic approach enables targeted experiments to determine how a gene is regulated or protein functions at an amino acid level. This strategy involves making targeted mutations to regulatory or coding regions of a gene in order to test the function of those specific regions of the gene or protein. In this way it is possible to test if specific components of a protein are responsible for a particular cellular mechanism. Of particular interest are the mechanisms that underlie vesicle fusion and neural transmission.

The biophysically challenging task of fusing vesicle and cell membranes together is accomplished by SNARE proteins (Jahn & Fasshauer, 2012). The Drosophila SNARE protein, neuronal-Synaptobrevin, is homologous to mammalian VAMP2 and is a neuronal protein important for synaptic transmission (Trimble et al., 1998; Di Antonio et al., 1993; Deitcher et al., 1998). The protein, anchored in neurotransmitter vesicle membranes, comes together with other SNARE proteins attached to the cells membrane. The proteins coil together forming a highly stable SNARE complex, pulling the membranes into close apposition (Sollner et al., 1993; Sutton et al., 1998). Upon receiving a calcium signal, a series of molecular events commence, SNARE complex formation completes, vesicle fusion occurs and neurotransmitter exits the neuron into the synaptic cleft (Huntwork and Littleton et al., 2007; Maximov et al., 2009). n-Syb contains a short, 10 amino acid, flexible region between the SNARE and transmembrane regions termed the juxtamembrane or linker region. This region contains a highly conserved tryptophan residue and several charged basic residues thought to interact with the membrane (Weimbs et al., 1998; Al-Abdul-Wahid et al., 2012). I hypothesized that this region of the molecule has a functional role in membrane fusion via tryptophan anchoring and force transduction through the region. Therefore, a reverse genetic approach would be appropriate for testing this hypothesis.
Deitcher et al., (1998), produced several $n$-syb mutants, including a null, which were utilized for this study. The null allele, termed $n$-syb$^{AF33B}$, was produced via imprecise P-element excision and was found to be embryonic lethal. A severe hypomorphic allele, $n$-syb$^{F33R}$, also embryonic lethal, was produced via P-element insertion. Therefore the coding region of the gene remained. Finally, the hypomorph, $n$-syb$^{Id}$, was produced via EMS mutation (ethyl methanesulfonate). This mutant produced low quantities of n-Syb (about 10%) and survives to adult, but exhibits severe motor impairment and a severely attenuated lifespan.

The first step in the reverse genetic approach was to create a transgenic wild-type version of the gene expressed in one of the aforementioned backgrounds. Previous attempts to rescue the $n$-syb null through the production of $UAS-n$-syb constructs did not demonstrate rescue (B. Stewart, personal communication; Bhattacharya et al., 2002). Such studies used constructs produced from the complementary DNA and were expressed with non-endogenous promoters using the UAS-Gal-4 system. Therefore, in this study we attempted to rescue the null allele by creating transgenic constructs from the genomic sequence, which included all introns, exons and the endogenous promoter. Wild-type transgenic constructs were found to restore $n$-syb hypomorphs to wild-type levels in all variables measured. This provided a model in which to test the effect of targeted mutation to the $n$-syb juxtamembrane region on the fly.

Materials and Methods

Cloning and Expression in S2 Cells

The production and mutation of genomic constructs is described in Chapter 4. Expression of transgenic protein from the wild-type and mutant constructs in Drosophila S2 cells is also described in chapter 4.
Transgenic Drosophila lines were created using P-element transformation (Genetic Services Inc., Sudbury, MA) using pCaSpeR2-n-syb\textsuperscript{WT}, pCaSpeR2-n-syb\textsuperscript{WW}, pCaSpeR2-n-syb\textsuperscript{AA}, pCaSpeR2-n-syb\textsuperscript{L6} and pCaSpeR2-n-syb\textsuperscript{L24}. Approximately 100 embryos were injected with each plasmid. All offspring were then assigned a number and crossed to \textit{w}\textsuperscript{1118} (white eye) flies. Offspring from this cross that successfully incorporated the transgenic plasmid displayed pigmented eyes (yellow to orange), because p-CaSpeR2 incorporated the \textit{white} gene. These flies were then assigned a letter and crossed to \textit{w}\textsuperscript{1118} to create a transgenic line (92 in total). Next, the chromosome into which the transgene inserted was determined by crossing each line to both a second (\textit{Gla/Cyo}) and third (\textit{TM2Ubx/TM6CSb}) balancer chromosome. Independent segregation from the balancer, either \textit{Cyo} (curly wings) or \textit{TM6CSb} (stubble bristles), indicated the location of the transgene. Transgenes located on the X chromosome were simply observed via gender segregation. While transgenes that inserted on the 2\textsuperscript{nd} chromosome were focused on due to their favorable genetic position for studying the effects of the transgenes on the 3\textsuperscript{rd} chromosome \textit{n-syb} alleles, an additional 6 X chromosome inserts and 6 3\textsuperscript{rd} chromosome inserts were also selected (see stock list). Table 2 summarizes the number of transgenic lines created. Production of a portion of these lines was accomplished with the assistance of undergraduate students: Farah Jazuli and Nicole Novroski.

Table 2: Summary of Transgenic Lines

<table>
<thead>
<tr>
<th>Transgenic Line</th>
<th>Number of Lines Established</th>
<th>Number with insertion on 2\textsuperscript{nd} chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{n-syb}\textsuperscript{WT}</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>\textit{n-syb}\textsuperscript{WW}</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>\textit{n-syb}\textsuperscript{AA}</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>\textit{n-syb}\textsuperscript{L6}</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>\textit{n-syb}\textsuperscript{L24}</td>
<td>18</td>
<td>7</td>
</tr>
</tbody>
</table>
Relative expression of transgenic protein was determined for most 2nd chromosome transgenics with western blot. Heads were manually separated from 12 flies of each genotype and ground separately from bodies in 30μl of lysis buffer with a protease inhibitor cocktail (Sigma-Aldrich) (300mM tris HCl pH 9, 100mM EDTA, 0.625% SDS, 5% sucrose). The samples were heated at 70°C for 15 minutes and 30μl of loading dye with fresh DTT added. Samples were boiled prior to loading in a 12% acrylamide gel. 25μl of each sample was loaded which was the equivalent of 5 heads. Western blot was performed and samples stained with 1:1000 anti-V5 antibody. Expression levels were then scored in a relative manner with 0 indicating no appreciable staining and 5 showing the darkest bands.

Test of rescue of n-syb\(^{AF33B}\)

To test rescue of the n-syb null mutant, 2nd and 3rd chromosome balancer stocks had to be made of all transgenic lines and of n-syb\(^{AF33B}\). First, a double balancer stock: Sp/Cyo;Ly/TM6ShTb was made. These flies were then crossed to the various second chromosome transgenic flies (n-syb\(^{TRANS}\)) to ultimately produce n-syb\(^{TRANS}/Cyo;Ly/TM6ShTb\). The double balancer stock was also used to produce Sp/Cyo;n syb\(^{AF33B}/TM6ShTb\). The now balanced version of the n-syb null could be crossed to the various transgenic lines (Sp/Cyo; n-syb\(^{AF33B}/TM6ShTb\) X n-syb\(^{TRANS}/Cyo;Ly/TM6ShTb\)) to produce a fly with both the null and transgenic genes (n-syb\(^{TRANS}/Cyo; n-syb^{AF33B}/TM6ShTb\)). These flies were then intercrossed to remove the Cyo balancer and produce a homozygous transgenic fly: n-syb\(^{TRANS}; n-syb^{AF33B}/TM6ShTb\). Each line was then intercrossed to determine if the transgene could functionally replace n-syb (Figure 18).

The homozygous n-syb null mutant: n -Syb\(^{AF33B}/n -syb^{AF33B}\), produces no n-Syb protein and is not viable beyond the embryo stage (Deitcher et al., 1998), thus if transgenic n-Syb functionally replaces endogenous n-Syb then the fly: n-syb\(^{TRANS};n -syb^{AF33B}/n -syb^{AF33B}\) might survive. The identity of this fly would be determined by loss of the TM6ShTb balancer. This balancer includes two dominant phenotypic markers (Tb: tubby larva; Sb: adults with short, stubble, bristles) to enable its identification in a particular line. Therefore, transgenic rescue of the n-syb null would produce normal pupa (loss of Tubby), and normal adults (loss of Stubble).
In addition, transgenic n-syb insertions on the X chromosome were tested with the same strategy (6 additional lines). In this case a double balanced stock of the X and 3rd chromosome was produced: FM7;Ly/TM6SbTb. This stock was used to make n-syb\textsuperscript{TRANS}/FM7;Ly/TM6SbTb and FM7; n-syb\textsuperscript{AF33B}/TM6SbTb. These lines were intercrossed to produce n-syb\textsuperscript{TRANS}/FM7; n-syb\textsuperscript{AF33B}/TM6SbTb and then n-syb\textsuperscript{TRANS}; n-syb\textsuperscript{AF33B}/TM6SbTb (the transgenic homozygote). Intercrossing this final line was used to determine if the transgene was able to rescue the null mutation again assessed by loss of Tubby and loss of Stubble (Figure 19).

Finally, wild-type transgenic insertions on the 3rd chromosome (6 lines) were tested for rescue of the n-syb null. Transgenic flies were crossed with n-syb null flies: n-syb\textsuperscript{TRANS}/TM6CSb X n-syb\textsuperscript{AF33B}/TM6SbTb and non-balanced females were collected (n-syb\textsuperscript{TRANS}/n-syb\textsuperscript{AF33B}). These females were then crossed to w\textsuperscript{1118} control flies, allowing recombination to occur. In Drosophila males do not undergo recombination, while females do. Males with the transgene, assessed by yellow eyes, and possibly the n-syb null (indicated by a question mark) were collected and tested. Individual males were first crossed to a third chromosome balancer fly: n-syb\textsuperscript{TRANS}, n-syb\textsuperscript{AF33B}/+ X DrPr/ TM6SbTb. The stock, now balanced, was tested for recombination with the n-syb null by crossing it to the n-syb null stock: n-syb\textsuperscript{TRANS}, n-syb\textsuperscript{AF33B}/ TM6SbTb X n-syb\textsuperscript{AF33B}/TM6SbTb. Lines that did not recombine n-syb\textsuperscript{AF33B} with the transgene were expected to produce progeny with a phenotypic ratio of: 1/3 non Stubble adults to 2/3 Stubble adults. Successful recombination of n-syb\textsuperscript{AF33B} with the transgene might be expected to produce all Stubble adults because the null mutation is homozygous lethal. This assay is of course imprecise because the presence of the wild-type transgene may rescue the n-syb null. Therefore, full rescue of the n-syb null would produce offspring in an identical ratio to a fly that did not recombine with the n-syb transgene at all. This theoretical result was however not observed and the assay proved effective with careful consideration of the possibility that the transgene might have allowed for partial or complete rescue of the n-syb null. 20 recombinant lines resulting from 4 of the 6 n-syb\textsuperscript{WT} 3rd chromosome insertion lines were produced and tested.

If secondary lethal mutations were present on the 3rd chromosome during the production of n-syb\textsuperscript{AF33B} then homozygous lethality would persist even if transgenic rescue
would otherwise be successful. Therefore, transgenic rescue was tested with $n$-syb$^{AF33B}$ expressed over a chromosome with a deficiency in the $n$-syb region ($Df(3L)BSC800$, $TM6Sb$; Bloomington Drosophila stock center, Indiana: #27372). The transgenic stock, $n$-syb$^{WT,40B}$; $n$-syb$^{AF33B}/TM6SbTb$, was crossed to the deficiency stock, $Df(3L)BSC800/TM6Sb$. Successful rescue adults would be observed by loss of the balancer phenotype Stubble.

Because attempts to rescue the $n$-syb null ($n$-syb$^{AF33B}$) may have resulted in larvae with motor deficits, it was hypothesized that those potential rescue larvae might require special rearing conditions. 35 2nd chromosome transgenic lines of the genotype $n$-syb$^{Trans};n$-syb$^{AF33B}/TM6SbTb$ were assessed by placing flies on different substrates or under different rearing conditions and screening pupae for non-Tubby rescue morphology. Substrates included standard Bloomingtons media (cornmeal 71.25g/L, agar 2.75g/L, yeast extract 16.9g/L, corn syrup 71.25mL/L, malt 45g/L, soy 9.75g/L, propionic acid 4.7mL/L), standard media plus banana baby food, standard media with 60% normal agar and yeast paste, whole milk based Drosophila media modified from Dason et al., (2010; cornmeal 90g/L, agar 2.5g/L, yeast extract 5g/L, corn syrup 110mL/L, whole milk powder 20g/L, propionic acid 4.7mL/L). Finally, flies were also raised on standard media in a 28ºC humidified incubator because 28ºC was the optimal temperature for rearing Drosophila and humidity was found to be the most important variable for survival of Synaptotagmin null mutants (Loewen et al., 2001).

Testing rescue of the $n$-syb null with $n$-syb transgenes under these conditions may prove successful because it has been demonstrated that under special rearing conditions the normally lethal synaptotagmin null could survive to the 3rd instar larval stage (Loewen et al., 2001). The flies were reared on molasses plates for 24hr. Embryos were lined up and a drop of yeast-water was applied to each embryo twice per day for three days. The plates were placed in large petri dishes with a wet paper towel to increase humidity and were maintained at room temperature.

Test rescue of $n$-syb$^{F33R}$

In the production of the $n$-syb null ($n$-syb$^{AF33B}$) Deitcher et al., (1998), inserted a P-element into the $n$-syb gene 150bp 5' of the ATG initiation site. It was found to be
homozygous lethal at the embryo stage but left the open reading frame of the gene intact. Therefore some n-Syb could potentially be produced and the allele may not represent a true null. Therefore, this allele may provide a more favorable background to test genetic rescue by n-syb transgenics. The strategy for testing rescue of n-syb\textsuperscript{F33R} was similar to testing transgenic constructs in the hypomorphic background (Figure 21). Double balanced transgenic flies: n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};Ly/TM6SbTb were crossed to double balanced n-syb\textsuperscript{F33R} flies: Sp/Cy\textsubscript{o};n-syb\textsuperscript{F33R}/TM6SbTb to produce n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{F33R}/TM6SbTb. This line, now with the transgene and n-syb\textsuperscript{F33R}, was crossed to the corresponding line with n-syb\textsuperscript{AF33B}: n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{AF33B}/TM6SbTb. Rescue flies, n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{F33R}/n-syb\textsuperscript{AF33B}, would be identified by absence of Tubby (larvae) or absence of Stubble (adults). Lines tested included 11 n-syb\textsuperscript{WT} lines on the 2\textsuperscript{nd} chromosome, 4 n-syb\textsuperscript{WT} lines on the X chromosome and 4 recombinant lines resulting from 3\textsuperscript{rd} chromosome transgenic lines.

Expression of n-syb transgenes in the n-syb hypomorphic background

After producing the n-syb null mutation (n-syb\textsuperscript{AF33B}) via P-element excision, Deitcher et al., (1998), produced additional mutations of n-syb via EMS mutation. The hypomorphic line (n-syb\textsuperscript{H}) which produces minimal quantities of n-Syb (about 10%), was viable to the adult stage, but was short lived and characterized by severe motor deficit. This background proved useful for testing transgenic lines where all lines were compared for their ability to restore lifespan and motor function. Lines that performed well were selected, and the effect of mutations to n-syb were then compared in the hypomorphic background.

Testing n-syb transgenes in the hypomorphic background was performed in a similar manner to testing rescue of the null (n-syb\textsuperscript{AF33B}) (see Figure 18). Double balanced transgenic flies: n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};Ly/TM6SbTb were crossed to double balanced n-syb\textsuperscript{H} flies: Sp/Cy\textsubscript{o};n-syb\textsuperscript{H}/TM6SbTb to produce n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{H}/TM6SbTb. This line, now with the transgene and the n-syb\textsuperscript{H} allele, was crossed to the corresponding line with the n-syb\textsuperscript{AF33B} allele: n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{AF33B}/TM6SbTb. Hypomorphs, n-syb\textsuperscript{TRANS}/Cy\textsubscript{o};n-syb\textsuperscript{H}/n-syb\textsuperscript{AF33B}, were identified by absence of Tubby (larvae) or absence of Stubble (adults) (Figure 21). All transgenic lines inserted on the second chromosome were tested: 11 n-syb\textsuperscript{WT}, 4 n-syb\textsuperscript{WW}, 13 n-
syb\textsuperscript{AA}, 2 n-syb\textsuperscript{L6} and 7 n-syb\textsuperscript{L24}. Six additional transgenic lines on the X chromosome were tested in the hypomorphic background by making double balanced flies combining the transgenes with n-syb\textsuperscript{H}: n-syb\textsuperscript{TRANS}/FM7;n-syb\textsuperscript{H}/TM6SbTb. Flies from these lines, homozygous for the transgene, were then crossed to the corresponding n-syb\textsuperscript{AF33B} line to produce transgenic flies in the hypomorphic background. Lines tested included: 4 n-syb\textsuperscript{WT} and 2 n-syb\textsuperscript{L24}. Finally, 9 recombinant lines resulting from 1 3rd chromosome n-syb\textsuperscript{WT} line were tested. Here, transgenic recombinants were simply crossed to the balanced n-syb\textsuperscript{H} stock and hypomorphs selected: n-syb\textsuperscript{TRANS}, n-syb\textsuperscript{AF33B}/TM6SbTb X n-syb\textsuperscript{H}/TM6SbTb.

Climbing ability of n-syb transgenics in the hypomorphic background

Adult flies resulting from transgenic expression of n-syb in the hypomorphic background (n-syb\textsuperscript{H}/n-syb\textsuperscript{AF33B}) were expected to differ in their ability to restore the characteristic motor deficit observed in the hypomorphs. To assay motor ability, adult flies were challenged with a simple climbing task. They were placed in a 100mL graduated cylinder (18cm) and tapped to the bottom initiating negative geotaxis (Ganetzky and Flanagan, 1978). Flies were run in 10 groups of 4 per genotype and timed for how long it took to reach the top of the cylinder. Flies that did not reach the top in the two minute limit were assessed based on maximum height achieved. Lines assayed included: the 2\textsuperscript{nd} chromosome transgenics, 11 n-syb\textsuperscript{WT}, 4 n-syb\textsuperscript{WW}, 13 n-syb\textsuperscript{AA}, 2 n-syb\textsuperscript{L6}, 7 n-syb\textsuperscript{L24}; X chromosome transgenics, 4 n-syb\textsuperscript{WT}, 4 n-syb\textsuperscript{L24}; and 9 recombinant lines resulting from 1 3\textsuperscript{rd} chromosome n-syb\textsuperscript{WT} insertion.

Lifespan of n-syb transgenics in the hypomorphic background

It was observed that n-syb hypomorphic adult flies died within a few days of hatching. Therefore the effect of the expression of various n-syb transgenes in the hypomorphic background was tested on lifespan. Flies were placed individually or in groups of up to 5, segregated by gender, in vials of standard media at room temperature (18-22 per genotype). Flies were placed in new vials if the condition of the food deteriorated. Vials were checked each day.
Survival to adult of various n-syb heterozygotes

The three n-syb alleles: null (n-syb^{AF33B}), severe hypomorph (n-syb^{F33B}), hypomorph (n-syb^{I4}), and over expression of transgenic n-syb: (n-syb^{WT40B}) and (n-syb^{WT54B}) were tested for survival to adult as heterozygotes. Those alleles with a morphological phenotype (n-syb^{F33R}, n-syb^{WT40B}, n-syb^{WT54B}) were assessed by simply producing heterozygous males, crossing those males to w^{1118} control females and scoring heterozygote and wild-type offspring. Those alleles without a morphological phenotype (n-syb^{AF33B} and n-syb^{I4}) were assessed in two stages. First balanced flies (n-syb/TM6SbTb) were crossed to w^{1118} control flies and the number of offspring with the n-syb allele (+/n-syb) were compared to the number with the balancer (+/TM6SbTb). Balancer chromosomes contain many mutations and thus were expected to affect survival to adult on their own. Therefore, heterozygous balancer males (+/TM6SbTb) were crossed to w^{1118} control females and offspring assessed. Now the ratio of balancer heterozygotes to n-syb heterozygotes could be compared to the ratio of balancer heterozygotes to wild-type flies. If the n-syb allele had no effect on survival, one would expect the same ratio of TM6SbTb/+ : n-syb/+ as +/TM6SbTb : +/-+. For each cross, 5 males and 5 females were combined for 3 days and flipped 4 times. This was replicated with 5 sets of flies for a total of 25 vials per cross. Adults from each vial were scored on day 15 and day 21. Because several of the alleles contain the white gene this may affect survival of w^{1118} flies thus confounding the results. Therefore one final test was conducted where heterozygous females for the white gene (w^{+}/w^{-}) were crossed to w^{1118} males (w^{-}/Y). It was hypothesized that this gene would not affect survival to adult and therefore a 1:1 ratio was predicted between heterozygotes with the white gene (w^{+}/w^{-} and w^{+}/Y) and homozygotes without (w^{-}/w^{-} and w^{-}/Y).
**Figure 18: Testing rescue of \( n\text{-syb}^{\Delta F33B} \) with 2\(^{nd}\) chromosome transgenic inserts**

Strategy to test transgenic rescue of the \( n\text{-syb} \) null. Using a double balancer stock, the 2\(^{nd}\) chromosome transgenic insertions and 3\(^{rd}\) chromosome \( n\text{-syb}^{\Delta F33B} \) were balanced (A). These lines were intercrossed producing a new line with both the transgene and null mutation (B). This line was intercrossed to produce a homozygous transgenic line and remove the Cyo balancer (C). Finally, this line was intercrossed to test if the transgene could rescue the null mutation (D) observed by loss of Tubby and Stubble, the phenotypic markers associated with the 3\(^{rd}\) chromosome balancer TM6.
\[ \begin{array}{ccc}
\text{n-syb Transgenic Stock} & \text{Double Balancer Stock} & \text{n-syb null Stock} \\
\text{♀ } n\text{-syb}^{\text{TRANS}}_{FM7} ; & \text{♂ } FM7 ; & \text{♀ } \pm ; \\
\text{♀ } n\text{-syb}^{\text{TRANS}}_{LY} ; & \text{♀ } n\text{-syb}^{\Delta F33B}_{LY} ; & \pm ; \\
\text{♀ } TM6SbTb & \text{♂ } TM6SbTb & + \text{ TM6SbTb}
\end{array} \]

**Strategy**

A. \( \frac{n\text{-syb}^{\text{TRANS}}_{FM7}}{\text{♀ } n\text{-syb}^{\text{TRANS}}_{LY} ; \text{♂ } FM7 ; \text{♀ } n\text{-syb}^{\Delta F33B}_{TM6SbTb}} \)

B. \( \frac{n\text{-syb}^{\Delta F33B}_{FM7}}{\text{♀ } n\text{-syb}^{\Delta F33B}_{TM6SbTb}} \)

C. \( \frac{n\text{-syb}^{\text{TRANS}}_{n\text{-syb}^{\Delta F33B}_{FM7}}}{\text{♀ } n\text{-syb}^{\Delta F33B}_{n\text{-syb}^{\Delta F33B}_{TM6SbTb}}} \)

D. \( \frac{n\text{-syb}^{\Delta F33B}_{n\text{-syb}^{\Delta F33B}_{FM7}}}{\text{♀ } n\text{-syb}^{\Delta F33B}_{n\text{-syb}^{\Delta F33B}_{TM6SbTb}}} \)

**Figure 19: Testing rescue of \( n\text{-syb}^{\Delta F33B} \) with X chromosome transgenic inserts**

Strategy to test transgenic rescue of the \( n\text{-syb} \) null. Using males from a double balancer stock, the X chromosome transgenic insertions and 3

\(^{\text{rd}}\) chromosome \( n\text{-syb}^{\Delta F33B} \) were balanced (A). Females from the transgenic line were crossed to males from the null line producing a new line with both the transgene and null mutation (B). This line was intercrossed to produce a homozygous transgenic line and remove the \( FM7 \) balancer (C). Finally, this line was intercrossed to test if the transgene could rescue the null mutation (D) observed by loss of \( Tubby \) and \( Stubble \), the phenotypic markers associated with the 3

\(^{\text{rd}}\) chromosome balancer \( TM6 \). It is important to note that only the female genotypes are depicted in steps B-D.
Figure 20: Testing rescue of \( n\text{-}syb^{AF33B} \) with 3rd chromosome transgenic recombinants

3rd chromosome \( n\text{-}syb \) transgenics were tested for rescue of the \( n\text{-}syb \) null by recombining the transgene with the \( n\text{-}syb \) allele. First, the transgenic line was crossed to the \( n\text{-}syb \) null (A) and female progeny without the balancer chromosome were selected. These flies were crossed to a control line, allowing recombination to occur (B). Male progeny from this cross were selected, assigned a number and crossed to a balancer line (C). Now balanced, each of these potential recombinant lines was tested by crossing to the \( n\text{-}syb \) null (D). Progeny from these crosses were scored. If \( n\text{-}syb^{AF33B} \) did not recombine with the transgene, then progeny should be distributed in proportions depicted in (E). If \( n\text{-}syb^{AF33B} \) did recombine with the transgene, then progeny should be distributed in proportions depicted in (F), although rescue of \( n\text{-}syb^{AF33B} \) by the transgene could produce some rescue larva or adults. Perfect rescue would appear phenotypically identical to the proportions depicted in (E), however perfect rescue was not expected. Recombinant transgenic lines were tested for rescue simply by intercrossing each line.
<table>
<thead>
<tr>
<th>$n$-syb Transgenic Stock</th>
<th>$n$-syb null Stock</th>
<th>$n$-syb Hypomorph Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$-syb&lt;sup&gt;TRANS&lt;/sup&gt;; Ly&lt;sup&gt;Cyo&lt;/sup&gt; TM6SbTb</td>
<td>$Sp$; $n$-syb&lt;sup&gt;AF33B&lt;/sup&gt;&lt;sup&gt;Cyo&lt;/sup&gt; TM6SbTb</td>
<td>$Sp$; $n$-syb&lt;sup&gt;I4&lt;/sup&gt;&lt;sup&gt;Cyo&lt;/sup&gt; TM6SbTb</td>
</tr>
</tbody>
</table>

**Strategy**

A. $n$-syb<sup>TRANS</sup>; Ly<sup>Cyo</sup> TM6SbTb X $Sp$; $n$-syb<sup>AF33B</sup><sup>Cyo</sup> TM6SbTb ➔ $n$-syb<sup>TRANS</sup>; $n$-syb<sup>AF33B</sup><sup>Cyo</sup> TM6SbTb

B. $n$-syb<sup>TRANS</sup>; Ly<sup>Cyo</sup> TM6SbTb X $Sp$; $n$-syb<sup>I4</sup><sup>Cyo</sup> TM6SbTb ➔ $n$-syb<sup>TRANS</sup>; $n$-syb<sup>I4</sup><sup>Cyo</sup> TM6SbTb

C. $n$-syb<sup>TRANS</sup>; $n$-syb<sup>AF33B</sup><sup>TM6SbTb</sup> X $n$-syb<sup>TRANS</sup>; $n$-syb<sup>I4</sup><sup>TM6SbTb</sup> ➔ $n$-syb<sup>TRANS</sup>; $n$-syb<sup>AF33B</sup><sup>n$-syb<sup>I4</sup></sup>

**Figure 21: Strategy for testing transgenic expression in the hypomorphic background**

To test the effect of $n$-syb transgenes in the hypomorphic background, the following strategy was used. First, each double balanced transgenic line was crossed to the balanced $n$-syb null to produce a line including the transgene and null mutation (A). This was repeated for each transgenic line with the balanced $n$-syb hypomorphic line (B). After producing lines that were homozygous for the transgene, the two lines were crossed together (C). Hypomorphs resulting from this cross lacked the balancer chromosome and thus balancer phenotypes: Tubby larva and Stubble adults. Note, this same strategy was used to test the near null $n$-syb allele $n$-syb<sup>F33R</sup>, but with $n$-syb<sup>F33R</sup> in place of $n$-syb<sup>I4</sup>. Also, a similar strategy was used to test $n$-syb transgenes expressed on the X chromosome, but with the first chromosome balancer FM7 in place of the second chromosome balancer Cyo.
Results

Transgenic Drosophila

Levels of transgenic n-Syb produced, assessed by western blot using an antibody against the V5 epitope tag, was found to vary among transgenic lines (Figure 22, Table 3).

Table 3: Expression of transgenic n-Syb

<table>
<thead>
<tr>
<th>Transgenic Line</th>
<th>Expression Level</th>
<th>Transgenic Line</th>
<th>Expression Level</th>
<th>Transgenic Line</th>
<th>Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n\text{-syb}^{WT\ 27A} )</td>
<td>1</td>
<td>( n\text{-syb}^{AA\ 5B} )</td>
<td>5</td>
<td>( n\text{-syb}^{L6\ 9A} )</td>
<td>2</td>
</tr>
<tr>
<td>( n\text{-syb}^{WT\ 27C} )</td>
<td>1</td>
<td>( n\text{-syb}^{AA\ 15B} )</td>
<td>0</td>
<td>( n\text{-syb}^{L6\ 23A} )</td>
<td>3</td>
</tr>
<tr>
<td>( n\text{-syb}^{WT\ 40A} )</td>
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<td>( n\text{-syb}^{AA\ 19B} )</td>
<td>0</td>
<td>( n\text{-syb}^{L24\ 14A} )</td>
<td>0</td>
</tr>
<tr>
<td>( n\text{-syb}^{WT\ 40B} )</td>
<td>4</td>
<td>( n\text{-syb}^{AA\ 27A} )</td>
<td>2</td>
<td>( n\text{-syb}^{L24\ 29A} )</td>
<td>1</td>
</tr>
<tr>
<td>( n\text{-syb}^{WW\ 7A} )</td>
<td>0</td>
<td>( n\text{-syb}^{AA\ 32A} )</td>
<td>1</td>
<td>( n\text{-syb}^{L24\ 32B} )</td>
<td>1</td>
</tr>
<tr>
<td>( n\text{-syb}^{WW\ 7B} )</td>
<td>2</td>
<td>( n\text{-syb}^{AA\ 40A} )</td>
<td>2</td>
<td>( n\text{-syb}^{L24\ 40A} )</td>
<td>5</td>
</tr>
<tr>
<td>( n\text{-syb}^{WW\ 35A} )</td>
<td>0</td>
<td>( n\text{-syb}^{AA\ 40B} )</td>
<td>2</td>
<td>( n\text{-syb}^{L24\ 44B} )</td>
<td>0</td>
</tr>
<tr>
<td>( n\text{-syb}^{WW\ 46A} )</td>
<td>0</td>
<td>( n\text{-syb}^{AA\ 45A} )</td>
<td>1</td>
<td>( n\text{-syb}^{L24\ 55A} )</td>
<td>3</td>
</tr>
<tr>
<td>( n\text{-syb}^{AA\ 2B} )</td>
<td>2</td>
<td>( n\text{-syb}^{AA\ 60A} )</td>
<td>0</td>
<td>( n\text{-syb}^{L24\ 57A} )</td>
<td>0</td>
</tr>
<tr>
<td>( n\text{-syb}^{AA\ 4B} )</td>
<td>0</td>
<td>( n\text{-syb}^{AA\ 60B} )</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Test rescue of \( n\text{-syb}^{AF33B} \)

Now that it had been established that transgenic protein was expressed in the transgenic fly lines, rescue of the embryonic lethal \( n\text{-syb} \) null could be tested. It was expected that genomic transgenes encoding wild-type \( n\text{-syb} \) including the endogenous promoter should restore the deficits imposed by the null mutation. All wild-type transgenic lines with insertions on X, 2\text{nd} or 3\text{rd} chromosome and all mutant transgenic lines with insertion on the 2\text{nd} chromosome were tested.
Genomic expression of transgenic n-syb was not found to rescue the embryonic lethal null mutation (n-syb^{ΔF33B}) to 3rd instar larval or adult stages. 6 lines with transgenic insertion on the X chromosome were tested: n-syb^{WT11H}, n-syb^{WT16}, n-syb^{WT45H}, n-syb^{WT45I}, n-syb^{L2424A}, n-syb^{L2445A} (see Figure 19 for cross). 36 lines with transgenic insertion on the 2nd chromosome were tested: n-syb^{WT11A}, n-syb^{WT11J}, n-syb^{WT15A}, n-syb^{WT27A}, n-syb^{WT27C}, n-syb^{WT40A}, n-syb^{WT40B}, n-syb^{WT45C}, n-syb^{WT45I}, n-syb^{WT54A}, n-syb^{WT54B}, n-syb^{WW7B}, n-syb^{WW35B}, n-syb^{WW46A}, n-syb^{AA2B}, n-syb^{AA4A}, n-syb^{AA4B}, n-syb^{AA5B}, n-syb^{AA15B}, n-syb^{AA19B}, n-syb^{AA27A}, n-syb^{AA32A}, n-syb^{AA40A}, n-syb^{AA40B}, n-syb^{AA45A}, n-syb^{AA60A}, n-syb^{AA60B}, n-syb^{L69A}, n-syb^{L616}, n-syb^{L2414A}, n-syb^{L2429A}, n-syb^{L2432B}, n-syb^{L2440A}, n-syb^{L2444B}, n-syb^{L2455A}, n-syb^{L2547A} (see Figure 18 for cross). Finally, of 6 wild-type transgenic lines with insertion on the 3rd chromosome, recombinants were successfully made from 4 lines and tested for rescue: n-syb^{WT11G} (5 recombinant lines), n-syb^{WT45E} (2 recombinant lines), n-syb^{WT59A} (9 recombinant lines), n-syb^{WT59B} (4 recombinant lines) (see Figure 19 for cross). However, none of these lines rescued the n-syb^{ΔF33B} to the larval or adult stage.

Due to the lack of rescue, I was concerned that perhaps there were other recessive lethal mutations present on the n-syb^{ΔF33B} chromosome. Therefore I designed a cross to test for rescue of the null allele over a chromosomal deficiency that removes the n-syb region. The wild-type transgenic line: n-syb^{WT40B}; n-syb^{ΔF33B}/TM6Sb Tb was crossed to a line carrying a deficiency which included the n-syb gene (Df(3L)BFC800/ TM6Sb; Bloomington's stock number: 27372). Successful rescue adult flies would display a non-Stubble phenotype: n-syb^{WT40B}+/; n-syb^{ΔF33B}/Df(3L)BFC800. However, no adult flies of this phenotype were observed (75 Stubble, 0 non-Stubble) indicating that rescue by transgenic n-syb was not prevented by unknown recessive lethal mutations.

Another possibility was that wild-type transgenes were producing a partial rescue, such that rescue to the embryonic or larval stage may occur, but not adult stage. To test this idea flies were reared on the following substrates: normal media supplemented with baby food, reduced agar (60% of normal) supplemented with yeast paste, crushed banana, whole milk based media and normal media at 28°C with increased humidity. While all 35 lines tested thrived on all media and in the 28°C humidified incubator, none showed transgenic rescue of n-syb^{ΔF33B}. It is possible that embryos possessing an otherwise lethal mutation from
motor impairment could be rescued if reared under special conditions as demonstrated by Loewen et al., (2001), with the synaptotagmin null mutant. However, application of a yeast solution directly to embryos did not result in rescue of \( n\text{-syb}^{\Delta F33B} \) by \( n\text{-syb} \) transgenics.

Despite the best attempts, it did not appear that the transgenic constructs would rescue \( n\text{-syb}^{\Delta F33B} \) mutants to adulthood. Therefore, I sought to determine if the transgenics would rescue less severe alleles.

**Test rescue of \( n\text{-syb}^{F33R} \)**

An additional embryonic lethal \( n\text{-syb} \) allele, \( n\text{-syb}^{F33R} \), was tested for rescue with wild-type \( n\text{-syb} \) transgenes. However, of 11 \( n\text{-syb}^{WT} \) lines on the 2\(^{nd}\) chromosome, 4 \( n\text{-syb}^{WT} \) lines on the X chromosome and 4 3\(^{rd}\) chromosome recombinant lines, none were found to rescue \( n\text{-syb}^{F33R}/n\text{-syb}^{\Delta F33B} \).

**Expression of \( n\text{-syb} \) transgenes in the \( n\text{-syb} \) hypomorphic background**

Expression of wild-type and mutant transgenic \( n\text{-syb} \) was tested in the \( n\text{-syb} \) hypomorphic background (\( n\text{-syb}^{I4}/n\text{-syb}^{\Delta F33B} \)). The hypomorph displayed severe motor impairment and reduced lifespan which was mitigated by many of the wild-type and mutant transgenic lines. Tested were: X chromosome inserts: \( n\text{-syb}^{WT\ 11H}, \ n\text{-syb}^{WT\ 16}, \ n\text{-syb}^{WT\ 45H}, \ n\text{-syb}^{WT\ 45I}, \ n\text{-syb}^{L24\ 24A}, \ n\text{-syb}^{L24\ 45A}, \) 2\(^{nd}\) chromosome inserts: \( n\text{-syb}^{WT\ 11A}, \ n\text{, syb}^{WT\ 11J}, \ n\text{-syb}^{WT\ 15A}, \ n\text{-syb}^{WT\ 27A}, \ n\text{-syb}^{WT\ 27C}, \ n\text{-syb}^{WT\ 40A}, \ n\text{-syb}^{WT\ 40B}, \ n\text{-syb}^{WT\ 45C}, \ n\text{-syb}^{WT\ 45J}, \ n\text{-syb}^{WT\ 54A}, \ n\text{-syb}^{WT\ 54B}, \ n\text{-syb}^{WW\ 7B}, \ n\text{-syb}^{WW\ 35B}, \ n\text{-syb}^{WW\ 46A}, \ n\text{-syb}^{AA\ 2B}, \ n\text{-syb}^{AA\ 4A}, \ n\text{-syb}^{AA\ 4B}, \ n\text{-syb}^{AA\ 5B}, \ n\text{-syb}^{AA\ 15B}, \ n\text{-syb}^{AA\ 19B}, \ n\text{-syb}^{AA\ 27A}, \ n\text{-syb}^{AA\ 32A}, \ n\text{-syb}^{AA\ 40A}, \ n\text{-syb}^{AA\ 40B}, \ n\text{-syb}^{AA\ 45A}, \ n\text{-syb}^{AA\ 60A}, \ n\text{-syb}^{AA\ 60B}, \ n\text{-syb}^{L6\ 9A}, \ n\text{-syb}^{L6\ 23A}, \ n\text{-syb}^{L24\ 14A}, \ n\text{-syb}^{L24\ 29A}, \ n\text{-syb}^{L24\ 32B}, \ n\text{-syb}^{L24\ 40A}, \ n\text{-syb}^{L24\ 44B}, \ n\text{-syb}^{L24\ 55A}, \ n\text{-syb}^{L24\ 57A}, \) and 9 3\(^{rd}\) chromosome recombinant lines resulting from \( n\text{-syb}^{WT\ 59A} \).
Climbing ability of n-syb transgenics in the hypomorphic background

Adult flies resulting from transgenic n-syb expressed in the hypomorphic background (n-syb^{WT}/n-syb^{ΔF33B}) were tested for motor deficits. The adult climbing assay enabled quantification of the motor impairment observed for the n-syb hypomorph (Figure 23). Almost all Oregon-R (Ore-R) control flies climbed to the top of the cylinder without delay while none of the hypomorphic flies were able to. However, some hypomorphic flies were able to right themselves and attempt to climb. Expression of the wild-type or mutant n-syb transgene restored motor function in many cases allowing flies to complete the climbing task (Figure 24).

It was predicted that the level of transgenic protein expression might be affected by where the wild-type or mutant n-syb transgene inserted into the chromosome, and that this level of transgenic protein expression might be related to how well a particular line rescued or restored function in the hypomorph. However, expression level of transgenic n-syb protein (see Figure 22, Table 3) did not correlate with restoration of climbing ability (Figure 23 and 24) when expressed in the hypomorphic background (Figure 25). Regulation of this gene may be complex and therefore a functional assay to directly show restoration of the hypomorphic phenotype was employed to allow for unbiased selection of transgenic lines for further study. Therefore, all transgenic lines were tested in the hypomorphic background with the climbing assay to select those for further study.

Suitable candidates for the wild-type transgenic control were determined by testing all available transgenic lines in the hypomorphic background with the climbing assay (Figure 23). Of the 16 lines tested, many restored the locomotor impairment observed in the hypomorph. Lines that did not perform well included: the 2^{nd} chromosome insert n-syb^{WT 40A}, the X chromosome inserts n-syb^{WT 11H}, n-syb^{WT 16A}, n-syb^{WT 45H}, n-syb^{WT 45I}, and the 9 recombinant lines of the 3^{rd} chromosome insert n-syb^{WT 59A}. The wild-type line selected, n-syb^{WT 40B}, showed a high proportion of completion (95%), quick time to top (15±1s) and large value for average height achieved (84±5% of top). While this line did show a slower time to top than Ore-R (9.5±1.2s; P<0.05, t-test) and lower height achieved than Ore-R (99±1%);
P<0.05, t-test) it was selected because it was the best of the wild-type transgenic lines that also contained a V5-epitope tag.

Transgenic lines resulting from each of the four mutations were observed to restore the hypomorph’s motor deficits (Figure 24). Exceptions included the second chromosome insertion of n-syb\textsuperscript{AA 5B} and the X chromosome insertions of n-syb\textsuperscript{L24 24A} and n-syb\textsuperscript{L24 45A}. Lines selected for further study were the following: n-syb\textsuperscript{WW 35B} (70\% complete in 11.7±0.6s), n-syb\textsuperscript{AA 60B} (97.5\%, 14.4±1.4s), n-syb\textsuperscript{L6 23A} (95\%, 14.6±0.8s) and n-syb\textsuperscript{L24 40A} (60\%, 19.3±3.6s).

Lifespan of n-syb transgenics in the hypomorphic background

The effect of the expression of various n-syb transgenes in the hypomorphic background was tested on lifespan, because it was observed that n-syb hypomorph adult flies died within a few days of hatching (Figure 26). These results mirror the climbing assay, where lines that did well in the climbing assay also exhibited a longer lifespan. Control Ore-R flies survived 92±7 days while the hypomorph survived for only 4.2±0.4 days. Several of the wild-type transgenic lines did restore normal lifespan to the hypomorph. Some of the lines that did not perform well included n-syb\textsuperscript{WT 40A} and the X and 3\textsuperscript{rd} chromosome inserts. Many of the transgenic lines carrying one of the four n-syb mutations showed a normal wild-type lifespan to the hypomorph. Exceptions included n-syb\textsuperscript{AA 5B}, n-syb\textsuperscript{L24 24A} and n-syb\textsuperscript{L24 45A}. Those lines selected for further study survived for (days): n-syb\textsuperscript{WT 40B} (59±6), n-syb\textsuperscript{WW 35B} (53±6), n-syb\textsuperscript{AA 60B} (87±8), n-syb\textsuperscript{L6 23A} (77±3) and n-syb\textsuperscript{L24 40A} (90±5).

Survival to adult of n-syb heterozygotes

The three n-syb alleles: null (n-syb\textsuperscript{AF33B}), severe hypomorph (n-syb\textsuperscript{F33R}), hypomorph (n-syb\textsuperscript{H}), and over expression of transgenic n-syb: (n-syb\textsuperscript{WT 40B}) and (n-syb\textsuperscript{WT 54B}) were tested for survival to adult as heterozygotes (Table 4).

For the null allele, n-syb\textsuperscript{AF33B}, there was no morphological phenotype in the heterozygote, therefore the ratio of heterozygous null to heterozygous balancers was compared to the ratio of wild-type to heterozygous balancers. If n-syb\textsuperscript{AF33B} had no effect on survival, one would expect the same ratio of n-syb\textsuperscript{AF33B}/+ : TM6SbTb/+ as +/- : TM6SbTb/+.
This was not the case however and the null heterozygote produced significantly fewer adults when compared to wild-type ($X^2=24.696$, df=1, $P<0.0001$).

The hypomorphic allele, $n$-$syb^{fs4}$, also did not display a morphological phenotype as a heterozygote and therefore the same strategy was employed as with the previous allele. In this case the number of hypomorphic heterozygote adults was not significantly different from wild-type adults ($X^2=0.001$, df=1, $P>0.05$).

The other alleles tested displayed a yellow eye phenotype and thus heterozygotes were compared directly to wild-type offspring. The near null, $n$-$syb^{F33R}$, did not produce a significantly different number of adults than wild-type ($X^2=0.571$, df=1, $P>0.05$). Unexpectedly, both of the wild-type transgenes expressed in the wild-type background (over expression of $n$-$syb$) produced a significantly greater number of heterozygous adults than their wild-type siblings ($n$-$syb^{WT\,40B}$, $X^2=9.199$, df=1, $P=0.002$; $n$-$syb^{WT\,54B}$, $X^2=14.648$, df=1, $P=0.0001$).

It could be that the results were confounded by differing levels of expression of the white gene, because the transgene is associated with the white gene to enable phenotypic identification of transgenics. However, a test cross indicated no significant difference in the survival of $w^+$ and $w^-$ flies ($X^2=0.004$, df=1, $P>0.05$).
Table 4: Survival to adult of heterozygous *n-syb* mutants

<table>
<thead>
<tr>
<th>Allele</th>
<th>Cross</th>
<th>Ratio</th>
<th>Result (# adults)</th>
</tr>
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<tr>
<td><em>n-syb</em>^{AF33B}</td>
<td><em>n-syb</em>^{AF33B}/TM6SbTb X <em>w</em>^{1118}</td>
<td><em>n-syb</em>^{AF33B}/+ : +/-TM6SbTb</td>
<td>1169 : 1062</td>
</tr>
<tr>
<td></td>
<td>+/-TM6SbTb X <em>w</em>^{1118}</td>
<td>+/- : +/-TM6SbTb</td>
<td>1012 : 742</td>
</tr>
<tr>
<td><em>n-syb</em>^{I4}</td>
<td><em>n-syb</em>^{I4}/TM6SbTb X <em>w</em>^{1118}</td>
<td><em>n-syb</em>^{I4}/+ : +/-TM6SbTb</td>
<td>975 : 827</td>
</tr>
<tr>
<td></td>
<td>+/-TM6SbTb X <em>w</em>^{1118}</td>
<td>+/- : +/-TM6SbTb</td>
<td>1116 : 981</td>
</tr>
<tr>
<td><em>n-syb</em>^{F33R}</td>
<td><em>n-syb</em>^{F33R}/+ X <em>w</em>^{1118}</td>
<td><em>n-syb</em>^{F33R}/+ : +/-</td>
<td>926 : 927</td>
</tr>
<tr>
<td><em>n-syb</em>^{WT40B}</td>
<td><em>n-syb</em>^{WT40B}/+ X <em>w</em>^{1118}</td>
<td><em>n-syb</em>^{WT40B}/+ : +/-</td>
<td>1028 : 895</td>
</tr>
<tr>
<td><em>n-syb</em>^{WT54B}</td>
<td><em>n-syb</em>^{WT54B}/+ X <em>w</em>^{1118}</td>
<td><em>n-syb</em>^{WT54B}/+ : +/-</td>
<td>843 : 693</td>
</tr>
<tr>
<td><em>w</em>+</td>
<td><em>w</em>+/<em>w</em> X <em>w</em>/Y</td>
<td><em>w</em>+(w+/w-, w+/Y) : w(w+/w-, w+/Y)</td>
<td>1087 : 1090</td>
</tr>
</tbody>
</table>
Figure 22: Expression of transgenic n-Syb in flies

Expression of wild-type and mutant transgenic n-syb observed via western blot. Each lane was loaded with and equivalent of 5 fly heads. Blots were stained with anti-V5 (arrow) and anti-β tubulin loading control (arrow head). Note (C) increased size of L24 insert is observed to run slower in gel: compare L24 55A to AA 2B. * indicates 3rd chromosome inserts.
A. Percent Complete

B. Time to top

C. Height achieved

Figure 23: Climbing ability of wild-type transgenics in the hypomorphic background

Climbing ability assessed in an 18cm, 100mL graduated cylinder for Ore-R, hypomorph and transgenic wild-type $n$-syb lines expressed in the hypomorphic background. The percentage of individuals that completed the task (A), time to top (s) of those that did complete the task (B), and total height achieved (percent top) for all individuals was measured (C). \( n = 40 \) flies per genotype. Note, error bars not included in (A) because this data represents the proportion of flies, out of 40, that completed the task and is thus essentially \( n=1 \).
Figure 24: Climbing ability of mutant transgenics in the hypomorphic background

Climbing ability assessed in an 18cm, 100mL graduated cylinder for mutant \( n-syb \) lines expressed in the hypomorphic background. The percentage of individuals that completed the task (A), time to top (s) of those that did complete the task (B), and total height achieved (percent top) for all individuals was measured (C). \( n = 40 \) flies per genotype. Note, error bars not included in (A) because this data represents the proportion of flies, out of 40, that completed the task and is thus essentially \( n=1 \).
Figure 25: Climbing ability vs. transgenic protein expression level

Climbing ability (percent complete) vs. relative transgenic expression level in wild-type and mutant *n-syb* transgenic lines. No correlation was observed between the amount of transgenic protein produced and climbing ability in the hypomorphic background. Note, error bars are not included on this figure because these data result from the proportion of flies of a given genotype that completed the climbing task and the relative expression estimated from a single western blot.
Figure 26: Lifespan of adult flies

Lifespan (days) observed in wild-type transgenic (A) and mutant transgenic (B) lines expressed in the hypomorphic background. A marked decrease in lifespan was observed in the hypomorph compared to the *Ore-R* control line (A). Several of the wild-type transgenic lines did restore lifespan of the hypomorph to wild-type levels. Although variable, several lines resulting from each of the 4 mutations were observed to restore lifespan to, or near, wild-type levels (B). n = 18 to 22 per genotype.
Discussion

While genomic expression of wild-type transgenic n-syb was not found to rescue the null (n-syb\(^{AF33B} / n-syb^{AF33B}\)) or near null (n-syb\(^{AF33R} / n-syb^{AF33B}\)) n-syb mutations, marked improvements were observed in the motor deficit and lifespan of the hypomorph (n-syb\(^{I4} / n-syb^{AF33B}\)). The hypomorph provided a useful background with which to test the effect of mutation to n-syb, because the wild-type transgene restored the hypomorphic phenotype to that of a wild-type control fly.

Flies expressing transgenic n-syb in the hypomorphic background were observed to vary in their ability to restore climbing ability, ranging from complete rescue to almost no effect. And while variation was also observed in the level of transgenic protein produced for different lines, no correlation was observed between level of protein expression and climbing ability. Therefore the climbing assay was used to test all transgenic lines and select those that performed the task well for further study. Interestingly, lines from each of the wild-type or four mutant lines were observed that performed well. It was not expected that all of the mutant lines would be able to restore the hypomorph’s motor impairment. Detailed analysis and discussion of differences between the mutant lines is presented in Chapter 4. Transgenic lines that were successful at the climbing assay also demonstrated a wild-type, or near wild-type lifespan. Therefore, the effects of n-syb transgenes were neither transient nor subtle, but restored flies from a nearly paralyzed condition that survived for only a few days to normal animals that lived for several months.

It was expected that the location of insertion of the transgene in the chromosome may affect its regulation and therefore, some inserts would be expected to perform better than others. However, because none of the transgenes were observed to rescue the n-syb null mutation, it is hypothesized that the original construct may be missing a critical component. The original construct included the genomic sequence of n-syb including the endogenous promoter. Confirmation of this was conducted by expressing the constructs, in P-elements, in Drosophila S2 cells. These neural progenitor cells, under direction of the endogenous promoter, did produce transgenic protein which was correctly oriented in the membrane. Therefore, the production of transgenic flies was initiated. Transgenic protein was observed to
be produced in flies in a wild-type background and in flies in a hypomorphic background. But why then did the transgene not rescue the null or severe hypomorphic mutations? It is likely that regulation of the production of this critical neuronal protein is complex and may include multiple regulator and enhancer elements. Perhaps these elements were not included in the genomic construct used to produce transgenic flies. If, for example, a regulator element responsible for production of n-Syb in early stages of development was not included, then a transgene expressed in the null would not survive. The hypomorph however, would produce enough n-Syb to allow the fly to progress through development to the adult stage providing the transgene with the opportunity to activate at a later stage. Investigation of this potential problem in gene regulation may be valuable in the study of how critical neuronal genes are regulated. These results also demonstrate that additional regions of the genome, beyond the known gene location, should be included when attempting genomic rescue.

Although levels of transgenic n-syb expression were not found to correlate with restoration of the hypomorphic phenotype, levels of endogenous n-syb expression do have a pronounced effect on the animal's survival. The extreme examples described thus far include the embryonic lethal null, which produces no n-Syb, the embryonic lethal severe hypomorph and the hypomorph, which produces 10% normal n-Syb. Heterozygotes of the null, severe hypomorph and hypomorph, appear normal upon gross inspection, however, the unexpected observation of a greater proportion of null heterozygotes was observed in combination with a wild-type transgene. This raised the question that the n-syb null heterozygote (n-syb$^{∆F33B}/+$) may be less viable than a wild-type fly and that two copies if n-syb are important for optimal survival.

During assembly of 3rd chromosomal transgenic recombinants, I observed an unexpected effect of the wild-type transgene on the n-syb$^{∆F33B}$ heterozygote. The null mutation was lethal in a homozygous form, but normal flies developed when the allele was expressed in a heterozygous form. However, I observed an increase in the ratio of flies expressing the wild-type transgene in the n-syb$^{∆F33B}$ heterozygous background vs. the n-syb$^{∆F33B}$ heterozygote alone. Therefore, perhaps the n-syb$^{∆F33B}$ heterozygote was not unaffected and exhibited decreased survival to adult which was mitigated by expression of transgenic n-syb. In fact, it has been demonstrated that the level of the expression of the
SNARE protein SNAP-25 was critical for normal synaptic function (Pozzi et al., 2008; Scullin et al., 2012). The cross: \( n\text{-}syb^{WT\ 59A}, n\text{-}syb^{AF33B}/TM6SbTb \times n\text{-}syb^{AF33B}/TM6SbTb \), was expected to produce the following genotypes at a 1:1 ratio: \( n\text{-}syb^{WT\ 59A}, n\text{-}syb^{AF33B}/TM6SbTb \) and \( n\text{-}syb^{AF33B}/TM6SbTb \) (a third genotype is possible if the transgene rescued the null, but was not observed in this case). The observed ratio of 471:405 was significantly different from the expected 1:1 ratio \( (X^2=4.973, \text{df}=1, P=0.02) \). This result indicated that the \( n\text{-}syb \) null heterozygote produced less adults than expected and introducing transgenic wild-type \( n\text{-}syb \) mitigated this deleterious effect.

Indeed, a decreased proportion of adult \( n\text{-}syb^{AF33B} \) heterozygotes was observed compared to their wild-type siblings. The near null \( (n\text{-}syb^{F33R}) \) and hypomorph \( (n\text{-}syb^{I4}) \) heterozygotes however did not produce ratios of offspring different from wild-type. Surprisingly, two different wild-type \( n\text{-}syb \) transgenes both produced a greater proportion of heterozygous offspring compared to their wild-type siblings. Overall, these results lend support to the claim that maintaining the endogenous SNARE protein level is critical to survival of the fly. Heterozygous flies with the null mutation or those over expressing transgenic \( n\text{-}syb \) appeared normal, but more careful observation revealed differences in survival to adult. Heterozygotes of another SNARE protein null mutant, SNAP-25, showed deficits in synaptic plasticity (Pozzi et al., 2008; Scullin et al., 2012), perhaps indicating an effect on learning and memory. Based on these results, more detailed electrophysiological and behavioural study of \( n\text{-}syb \) heterozygotes would be warranted to determine the effect of more subtle changes in \( n\text{-}Syb \) levels.

Although transgenic rescue of the \( n\text{-}syb \) null was unsuccessful, transgenic expression of \( n\text{-}syb \) in the hypomorphic background eliminated the hypomorphic phenotype and provided a model in which to test the effect of mutation of \( n\text{-}syb \). Control and regulation of the timing of expression and level of expression of \( n\text{-}Syb \) appears critical. Further examination of the regulation of \( n\text{-}syb \) and how the level of \( n\text{-}Syb \) affects the organism would be valuable in gaining fundamental understanding of basic neurophysiological principles.
Chapter 4 Investigation of the Juxta-membrane Region of neuronal-Synaptobrevin in Synaptic Transmission at the *Drosophila* Neuromuscular Junction

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**Author Contributions:** Experiments designed and conducted by C DeMill. Linker clones and expression in S2 cells was completed by X Qiu. The majority of the anatomical analysis was performed by A Bolotta.
Abstract

The juxtamembrane region of the *Drosophila* SNARE protein, neuronal-Synaptobrevin (n-Syb), was tested for its role in synaptic transmission using transgenes expressed in an *n-syb* hypomorphic background. Hypomorphic flies displayed severe motor inhibition, limited lifespan, reduced evoked junctional potentials (EJPs), decreased synchronicity in EJP time to peak and potentiation with 10Hz stimulation. All of these deficits were restored to wild-type levels with the expression of wild-type transgenic *n-syb*, regulated by the endogenous promoter (*n-syb*WT). To investigate the role of the *n-syb* juxtamembrane region on synaptic transmission, mutations were engineered into the transgene. We hypothesized that highly conserved interfacial tryptophan residues play a functional role in synaptic transmission by anchoring the molecule to the membrane. Therefore transgenic mutants with one additional tryptophan (*n-syb*WW) or mutation of tryptophan to alanine (*n-syb*AA) were tested. While *n-syb*WW resembled *n-syb*WT in all variables listed, *n-syb*AA exhibited decreased EJP amplitude, synchronicity and quantal content. To determine whether the *n-syb* linker region is important for transduction of force arising from SNARE complex assembly during membrane fusion we introduced short 6 amino acid (*n-syb*L6) or long 24 amino acid (*n-syb*L24) flexible linkers into the *n-syb* transgene. We observed a reduced EJP amplitude in *n-syb*L6 and decreased quantal content and an effect on the readily releasable and recycling vesicle pools in both *n-syb*L6 and *n-syb*L24. In conclusion, mutation of the juxtamembrane region of *n-syb* deleteriously affected synaptic transmission at the *Drosophila* neuromuscular junction.

Introduction

Chemical synaptic transmission requires fusion of neurotransmitter containing vesicles in a highly regulated and temporally efficient manner. This biophysical challenge is accomplished with a set of SNARE proteins (Weber *et al*., 1998): Syntaxin-1A, anchored to the plasma membrane (Oyler *et al*., 1989); SNAP-25 attached to the plasma membrane via a palmitoyl link (Bennett *et al*., 1992); and VAMP (Vesicle Associated Membrane Protein aka Synaptobrevin), anchored to the vesicle membrane (Trimble *et al*., 1988). Membrane fusion
occurs via formation of the well characterized, highly stable, SNARE complex where the
SNARE motifs of Syntaxin, Synaptobrevin and two from SNAP-25 associate from N-
(membrane distal) to C- (membrane proximal) termini to form a 4-helix coiled-coil (Sollner et
al., 1993; Sutton et al., 1998). The complex is thought to remain in a partially assembled
state, primed and ready for fusion (Hua and Charlton, 1999), but prevented from full
assembly by the fusion clamp: complexin. The clamp is removed upon action potential
evoked calcium entry and full assembly of the SNARE complex resulting in membrane fusion
(Huntwork and Littleton, 2007; Maximov et al., 2009).

Conserved membrane proximal tryptophan residues found in VAMPs (Weimbs et al.,
1998), have been hypothesised to play a role in membrane anchoring and fusion. SNARE
complex assembly may produce strain on opposing membranes potentially causing lipid
mixing and facilitating membrane fusion (Sutton et al., 1998). Strong anchoring would likely
be a requirement of such a mechanism. In an NMR study, we observed reduced membrane
anchoring with mutation of tryptophan to alanine in Drosophila neuronal-Synaptobrevin (n-
Syb) (Al-Abdul-Wahid et al., 2012). Other studies of fusion with mutations to these residues
have produced mixed results: decreased secretion from PC12 cells (Quetglas et al., 2002;
tryptophan 89 and tryptophan 90 to alanine), increased liposome fusion (Kweon et al., 2003;
tryptophan 89 and tryptophan 90 to serine), and no effect on liposome fusion (Siddiqui et al.,
2007; tryptophan 89 and tryptophan 90 to serine and deletion). However, neural models of
Ca\(^{2+}\) evoked transmitter release have demonstrated reduced amplitude evoked potentials and
increased miniature frequency in cultured mouse pyramidal neurons (Maximov et al., 2009;
tryptophan 89 and tryptophan 90 to alanine). In chromaffin cells reduced vesicle priming
inhibiting the exocytotic burst phase was observed (Borisovska et al., 2012; tryptophan 89
and tryptophan 90 to alanine; tryptophan 89 and tryptophan 90 to serine).

Here we test the hypotheses that conserved interfacial tryptophan residues in the
juxtamembrane region play a functional role in synaptic transmission through strong
membrane anchoring using transgenic expression of n-syb in an n-syb hypomorphic
background (Deitcher et al., 1998). Transgenic expression in the hypomorphic background is
a useful model because the animals survived until the adult stage enabling the collection of
behavioural, morphological and electrophysiological data from the larval and adult stages.
Hypomorphic flies displayed severe motor inhibition, limited lifespan, and synaptic
physiological deficits that were all corrected to wild-type levels with expression of the wild-type transgene (n-syb\textsuperscript{WT}) driven by the endogenous promoter. The role of tryptophan in the juxtamembrane region was tested by producing transgenic mutants with the addition (n-syb\textsuperscript{WW}) or removal (n-syb\textsuperscript{AA}) of one tryptophan residue while the role of the juxtamembrane region in bringing membranes into close apposition and transducing force was tested by producing mutants with a short, flexible, 6 amino acid linker (n-syb\textsuperscript{L6}) and a long 24 amino acid linker (n-syb\textsuperscript{L24}) inserted into the juxtamembrane region.

**Materials and Methods**

**Cloning**

Transgenic constructs of the n-syb gene were created by PCR amplifying the entire genomic sequence (6 Kb) from the corresponding BAC clone (RP98 17K18, Children’s Hospital Oakland Research Institute). The high fidelity DNA polymerase Phusion was used to reduce error (Finnzymes). BglII restriction sites incorporated at each end were used to clone the fragment into the P-element pCaSpeR2 (Thummel and Pirrotta, 1992). Due to the large size of the plasmid (13.5 Kb), site directed mutagenesis was performed on a 2 Kb portion of the gene sub cloned into pBS (pBlueScript, Stratagene) to produce the following: leucine 90 mutated to tryptophan (WW); tryptophan 89 and leucine 90 mutated to alanine (AA) and insertion of a linker between arginine 86 and lysine 87, GGTGGS (L6) (Figure 27, Table 5; see Deak et al., 2006). The segment of the gene containing the mutated portion was cut out from pBS and replaced in pCaSpeR2 using the endogenous restriction sites: NheI and MfeI. The L6 mutant was then digested with KpnI, dephosphorylated and ligated with an oligo incorporating a KpnI overhang encoding the following amino acid sequence: GGTGGSGGGS. A double insertion of the oligo created the L24 linker mutant: GGTGGSGGGSGTGGGSGGSGGGTGGGS. A V5 tag, GKIPNPLLGLDST, was included at the C terminal end of the 192 amino acid protein using site directed mutagenesis. Identity of constructs was confirmed by restriction analysis and sequencing (ACGT corporation, Toronto, ON). Primers and oligo nucleotides were from Sigma Aldrich while enzymes were from New England Biolabs.
Cell Expression

*Drosophila* S2 cells were employed to ensure protein expression, correct membrane integration and trafficking of \(n\)-*syb* transgenic protein. S2 (Schneider 2) cells, grown in CCM3, were transfected (using Cellfectin, Invitrogen) with the following plasmids: pCaSpeR2-n-syb\(^{WT}\), pCaSpeR2-n-syb\(^{AA}\), pCaSpeR2-n-syb\(^{L6}\) and pCaSpeR2-n-syb\(^{L24}\). Cells were allowed to grow for two days with protein expression driven by the endogenous promoter. Cells were then fixed in 4% paraformaldehyde in PBS for 10 minutes and stained with anti-V5 antibody (1:2000) for 1 hour in PBS plus 1% triton-X. An additional pCaSpeR2-n-syb\(^{WT}\) preparation was stained without Triton-X to avoid cell permeablization and determine the orientation of the V5 tag which was expected to be intravesicular / extracellular.

**Table 5: \(n\)-*syb* constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Purpose</th>
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<tr>
<td>pCaSpeR2-n-syb(^{WT})</td>
<td>Test rescue of hypomorph, control transgenic</td>
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<tr>
<td>pCaSpeR2-n-syb(^{WW})</td>
<td>Addition of tryptophan</td>
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<tr>
<td>pCaSpeR2-n-syb(^{AA})</td>
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<td>pCaSpeR2-n-syb(^{L24})</td>
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<td></td>
<td>83-KLKR(GGTGGS GGSGTGGS GGTGGS)KFWLQNLKMM-96</td>
</tr>
</tbody>
</table>

Drosophila Genetics

Transgenic *Drosophila* lines were created (92 total) using P-element transformation (Genetic Services Inc., Sudbury, MA) with the following plasmids: pCaSpeR2-n-syb\(^{WT}\), pCaSpeR2-n-syb\(^{WW}\), pCaSpeR2-n-syb\(^{AA}\), pCaSpeR2-n-syb\(^{L6}\) and pCaSpeR2-n-syb\(^{L24}\). Lines
with insertion on the second chromosome, determined using standard *Drosophila* genetics, were selected for further characterization (38 lines). Transgenic lines were not found to rescue the *n-syb* null mutation in either the homozygous form (*n-syb*$_{AF33B}$/*n-syb*$_{AF33B}$) or with the null expressed over the deficiency (*n-syb*$_{AF33B}$/*Df(3L)BSC800, TM6Sb*) (Bloomington *Drosophila* stock center, Indiana: #27372), therefore expression of transgenic wild-type and mutant *n-syb* were tested in the hypomorphic background (*n-syb*$_{I4}$/*n-syb*$_{AF33B}$). This was accomplished by crossing flies with the transgene (2nd chromosome) and either the hypomorphic or null mutation (3rd chromosome) balanced over TM6ShTb to each other: *n-syb*$_{TRANS}$/*n-syb*$_{I4}$/TM6ShTb X *n-syb*$_{TRANS}$/*n-syb*$_{AF33B}$/TM6ShTb. Transgenic hypomorph flies (*n-syb*$_{TRANS}$/*n-syb*$_{I4}$/*n-syb*$_{AF33B}$) were observed by loss of the balancer phenotypes: Tubby (larval) or Stubble (adult). Relative expression of transgenic protein was determined for each line with western blot. In addition, RNAi interference of *n-syb* was tested using the UAS-Gal4 system (Brand and Perrimon, 1993). RNAi flies, UAS-*n-syb*-RNAi, were obtained from the Vienna *Drosophila* RNAi centre and crossed to the pan neural driver ELAV-Gal4.

It was found that expression level of transgenic protein did not correlate with rescue of the hypomorphic phenotype in wild-type or mutant transgenic lines. Therefore a climbing assay was used to assess the motor impairment of the transgenic lines. Flies (10 groups of 4 per line) were placed in a 100mL graduated cylinder (18cm) and tapped to the bottom to initiate negative geotaxis (Ganetzky and Flanagan, 1978). The time to top or maximum height achieved within 2 minutes, the maximum time allotted, was determined. One representative line from each of the original 5 transgenic constructs was selected for further study based on performance of this test (*n-syb*$_{WT-40B}$; *n-syb*$_{WW-35B}$; *n-syb*$_{AA-60B}$; *n-syb*$_{L6-23A}$; *n-syb*$_{L24-40A}$).

The rate of development was determined for these lines. Five males and 5 females were placed together in 5 successive vials for 24hrs. Vials were scored every 24hrs. The test was repeated three times with different sets of flies. Time to 3rd instar larvae emerging, pupal formation and adult hatching was assessed. The proportion of rescue/normal pupae to tubby pupae and rescue/normal adults to stubble adults was determined. Lifespan was determined by placing flies individually or in groups of up to 5, segregated by gender, in vials of standard media at room temperature (18-22 per genotype). Vials were assessed each day and changed as necessary.
Neuromuscular Junction Morphology

Wandering 3rd instar larvae were dissected in HL3 (Stewart et al., 1994) and fixed for 10 minutes in 4% paraformaldehyde in PBS. Larvae were washed in PBT (PBS with 0.1% triton X) then blocked with goat serum (0.05v/v) for 1hr. Preparations were then incubated with 1:1000 FITC conjugated goat anti HRP for 1hr, washed in PBT and mounted on a slide with vectashield. NMJs on muscles 6/7 in segments 3 to 5 were imaged on a Zeiss confocal microscope (LSM510). Image stacks were flattened and analyzed with ImageJ. No more than two NMJs were imaged per larva for a total of 10-12 NMJs per genotype. The average NMJ length, number of branches and number of boutons was determined for each genotype.

Electrophysiology of Synaptic Transmission

Wandering 3rd instar larvae were selected for electrophysiological characterization. Intracellular recordings from muscle 6 in segments 3 and 4 were made using a glass micro electrode pulled to a diameter with a resistance of approximately 20MΩ when filled with 3M KCl. Muscle cells with a membrane potential more hyperpolarized than -60mV and a resistance of more than 5MΩ, determined with a 1nA pulse, were selected for study. Recordings were made in standard HL3 (Stewart et al., 1994) with 1.0mM CaCl₂ except where indicated. Initially, 2 minute recordings were taken from each muscle cell to observe the frequency and amplitude of spontaneous release. Preparations were then stimulated by shocking the nerve running to the corresponding hemi-segment 16 times at 0.1 Hz to determine the average evoked EJP potential. Immediately following this, a 5 minute train of stimuli at 10Hz was delivered followed by 16 stimuli at 0.1Hz. Only recordings that consisted of faithful stimulation of both axons leading to the hemi-segment for the entire 5 minute train were included in analysis of 10Hz stimuli. EJP amplitudes were corrected for non-linear summation according to McLachlan and Martin (1981; calculated potential = recorded potential / (1 - 0.25 * (recorded potential / membrane potential))). Synchronicity of release was determined from the 16 stimuli at 0.1Hz and the first 16 stimuli at 10Hz. The time to peak from an arbitrary point was determined for each EJP in the set and then variance calculated for each set of stimuli.
Figure 27: n-Syb and mutations

This schematic depicts neuronal-Synaptobrevin, a 192 amino acid VAMP responsible for neurotransmitter vesicle fusion in the *Drosophila*. Point mutations were made to the highly conserved juxtamembrane region by either adding or removing one tryptophan residue. Flexible linkers were introduced between the juxtamembrane region and SNARE motif. Regions are: **SNARE motif** (green), **juxtamembrane region** (blue), transmembrane domain (black), **intravesicular region** (purple).
Quantal content of evoked release was determined using the method of failures. The preparation was stimulated 500 times at 1Hz in low calcium (0.2mM CaCl₂) (n=6). The number of failures was determined as the number of stimuli falling below 0.2mV and the following equation applied: Quantal content = ln(N₀/N). Where N₀ was the total number of trials and N was the number of failures (Petersen et al., 1997).

Analysis

Most data sets were compared in two ways to reflect the fact that two control lines were used in this study: Oregon-R (Ore-R) and n-sybWT transgenic hypomorphs. First, differences between all groups (Ore-R, hypomorph, n-syb RNAi and the 5 transgenic hypomorphs) were assessed using a one way ANOVA and compared with the Dunnett's multiple comparison test to the Ore-R control line. Second, differences within the transgenic hypomorphic lines (n-sybWT, n-sybWW, n-sybAA, n-sybL6, n-sybL24) were also assessed using a one way ANOVA and compared with the post hoc Dunnett's multiple comparison test to the n-sybWT transgenic control line. Graphs were produced using MS Excel and statistics calculated using Graph Pad.

Results

Expression of n-syb in S2 cells

Transgenic n-syb constructs: pCaSpeR2-n-sybWT, pCaSpeR2-n-sybAA, pCaSpeR2-n-sybL6 and pCaSpeR2-n-sybL24, were found to produce membrane bound protein in Drosophila S2 cells when stained with an antibody for the V5 epitope tag (Figure 28). Because the V5 tag was located on the intra-vesicular portion of the protein, it was expected to also be found extracellularly after vesicle exocytosis. Indeed, the V5-tag was found extracellularly on S2 cells expressing pCaSpeR2-n-sybWT stained with anti-V5, but without the cell permeabilizing detergent, Triton-X. Therefore, genomic constructs of wild-type and mutant n-syb were able to produce transgenic protein that integrated and oriented correctly in the membrane.
Figure 28: Expression of transgenic n-Syb in Drosophila S2 cells

(A) Drosophila S2 cells expressing transgenic constructs: pCaSpeR2-n-syb\textsuperscript{WT}, pCaSpeR2-n-syb\textsuperscript{AA}, pCaSpeR2-n-syb\textsuperscript{L6} and pCaSpeR2-n-syb\textsuperscript{L24} stained with phalloidin (green) and anti-V5 (red). Transgenic protein appears to integrate and localize to the membrane. Scale bar indicates 10 μm. (B) schematic showing that the intravesicular location of the V5 epitope tag should be found extracellularly after vesicle exocytosis if the protein is in the correct orientation. (C) S2 cells expressing pCaSpeR2-n-syb\textsuperscript{WT} stained with anti-V5 without the membrane permeabilizing detergent, Triton-X, demonstrate correct orientation of the transgenic protein.
Expression of n-syb transgenes in an n-syb hypomorphic background

Expression of protein in transgenic hypomorphic adults ($n$-syb$^{\text{TRANS}}$, $n$-syb$^{I4}$/n-syb$^{\Delta F33B}$) was determined in samples of crushed fly heads via Western blot for all lines with insertion on the 2nd chromosome (38 total) and shown for those lines selected for further study (Figure 29); $n$-syb$^{\text{WT-40B}}$, $n$-syb$^{\text{WW-35B}}$, $n$-syb$^{\text{AA-60B}}$, $n$-syb$^{\text{L6-23A}}$, $n$-syb$^{\text{L24-40A}}$). Lines selected for further study are simply referred to by their mutation: where $n$-syb$^{\text{WT-40B}}$ is indicated as $n$-syb$^{\text{WT}}$. Transgenic protein expression was found to express predominantly in samples prepared from fly heads vs. fly bodies indicating primary neural expression. Interestingly, the level of transgenic protein expression was not found to correlate with the level rescue of motor impairment and reduced lifespan observed in the hypomorph. Bands observed corresponded to the expected size of n-Syb (20KDa) for n-Syb$^{\text{WT}}$, n-Syb$^{\text{WW}}$, and n-Syb$^{\text{AA}}$ while bands indicating larger proteins were found for n-Syb$^{\text{L6}}$ and n-Syb$^{\text{L24}}$ as expected.

Climbing assay

The ability of a fly to climb a glass graduated cylinder after being tapped to the bottom was used to assess motor impairment and to select transgenic lines for further characterization. Wild-type Ore-R flies climbed quickly to the top of the cylinder, while n-syb hypomorphs and n-syb-elav-RNAi flies attempted, but were unable to climb the cylinder (Figure 30). Overall, hypomorphic flies expressing transgenic n-syb (wild-type or mutant) were able to perform the task well but with some differences. While most n-syb$^{\text{WT}}$, n-syb$^{\text{AA}}$ and n-syb$^{\text{L6}}$ flies were able to complete the task, only 70% of n-syb$^{\text{WW}}$ and 60% of n-syb$^{\text{L24}}$ made it to the top in the allotted 2 minutes (Figure 30A). Of those flies that did climb to the top, no difference was observed in the time taken when comparing the transgenic mutant lines to the transgenic wild-type control (Figure 30B, ANOVA, P>0.05). However, for total height achieved, a slightly higher value was observed in n-syb$^{\text{AA}}$ compared to n-syb$^{\text{WT}}$ (Figure 30C, ANOVA, Dunnnett's multiple comparison, P=0.001). In summary, no major differences were observed between the different transgenic lines' ability to rescue the hypomorphs' motor impairment.
Figure 29: Expression of transgenic n-Syb

Western blot stained with anti-V5 and anti-β-Tubulin of severed heads (A), or bodies (B), 5 per lane. n-Syb (20KDa) is indicated by the arrow while the loading control, β-Tubulin (50KDa), is indicated by the arrow head. Differential expression of the transgenic protein was observed in different transgenic lines. The slightly larger size of n-Syb$^{L6}$ and n-Syb$^{L24}$ is observed in blot (A). Expression of transgenic protein is not observed in (B, fly bodies) indicating a primary neural expression pattern for the transgenic proteins.
Development

The rate of development and proportion of rescue of hypomorphic larvae and adults was assessed. The time from adding a group of flies to a vial to the emergence of wandering 3\textsuperscript{rd} instar larva was found to be longer for several of the transgenic lines in the hypomorphic background ($n$-syb\textsuperscript{WT}, $n$-syb\textsuperscript{AA}, $n$-syb\textsuperscript{L6}, $n$-syb\textsuperscript{L24}) compared to the Ore-R control line (Figure 31A, ANOVA, Dunnett's multiple comparison, P< 0.01). The proportion of rescue larva resulting from the test cross did not differ significantly between the hypomorphs and the transgenic lines in the hypomorphic background (Figure 31B, ANOVA, P>0.05). The length of time for pupal development did not differ between Ore-R control, hypomorph, or transgenic lines in the hypomorphic background (Figure 31C, ANOVA, P>0.05). The time to adult hatching was found to be significantly shorter for two transgenic lines in the hypomorphic background, $n$-syb\textsuperscript{WW} and $n$-syb\textsuperscript{L24} (11.0±0.2 and 11.6±0.5 days respectively) compared to Ore-R controls (13.2±0.1 days) (Figure 31D, ANOVA, Dunnett's multiple comparison, P=0.01), but did not differ among the transgenic mutant lines when compared to the transgenic control line (ANOVA, P>0.05). In summary, development of flies expressing wild-type or mutant transgenic $n$-syb in the hypomorphic background showed somewhat slower development to the 3\textsuperscript{rd} instar larval stage and an overall faster time to adult hatching. However, differences in development were not observed when comparing mutant transgenic hypomorphs to the wild-type transgenic hypomorph.

Hypomorphs had a much shorter lifespan (4.2±0.4 days) compared to Ore-R controls (92±7 days) which was restored with transgenic expression of $n$-syb\textsuperscript{WT} (59±6.3 days) (Figure 32). Reduced $n$-syb expression, observed in the $n$-syb RNA\textsuperscript{i} line also showed reduced lifespan similar to hypomorphs (5.6±0.5 days). Among transgenic lines in the hypomorphic background, two mutant lines, $n$-syb\textsuperscript{AA} and $n$-syb\textsuperscript{L24}, showed significantly longer life spans (84±7.1 and 89.6±5.0 days respectively) than $n$-syb\textsuperscript{WT} (ANOVA, Dunnett's multiple comparison, P<0.001). Therefore, expression of wild-type or mutant $n$-syb in the hypomorphic background dramatically improved lifespan to near control (Ore-R) levels compared to the hypomorph alone and thus mutations to the juxtamembrane region of $n$-syb did not deleteriously affect lifespan.
Motor ability was assessed by tapping adult flies (10 groups of 4 per genotype) to the bottom of a glass graduated cylinder (18cm) initiating negative geotaxis. Percent to complete the task (A), average time to reach the top (B) and average height achieved in the 2 minutes allotted (C) were measured. Hypomorph and n-syb RNAi lines were unable to complete the task, but expression of wild-type or mutant transgenic n-syb in the hypomorphic background restored climbing ability. n-syb\textsuperscript{WW} and n-syb\textsuperscript{L24} showed a decreased percentage complete (A) compared to n-syb\textsuperscript{WT}. Of flies that did complete the task, there was no difference in time to top (B, ANOVA, P>0.05). For total height achieved (C), a slight improvement was observed in n-syb\textsuperscript{AA} compared to n-syb\textsuperscript{WT}, ANOVA, Dunnett’s multiple comparison, P=0.001). Note, error bars are not included in (A) because this data results from the proportion of the 40 flies that completed the task.
A. Larval Hatching

B. Proportion Rescue

C. Pupal Development

D. Adult Hatching

**Figure 31: Development**

Rate of development of hypomorphic flies arising from crosses of 5 males and 5 females ($n$-$syb^{TRANS};n$-$syb^{AF33B}/TM6ShTb \times n$-$syb^{TRANS};n$-$syb^{I4}/TM6ShTb$). Time to 3rd instar larva emerging was longer for $n$-$syb^{WT}$, $n$-$syb^{AA}$, $n$-$syb^{L6}$ and $n$-$syb^{L24}$ compared to Ore-R (A, ANOVA, Dunnett’s multiple comparison, P<0.01). Proportion of hypomorphic larva (full rescue = 33%) resulting from the cross did not differ significantly between hypomorphs and the transgenic lines in the hypomorphic background (B, ANOVA, P>0.05). Length of time for pupal development did not differ between Ore-R control, hypomorph, or transgenic lines in the hypomorphic background (C, ANOVA, P>0.05). Adult hatching was faster for $n$-$syb^{WW}$ and $n$-$syb^{L24}$ compared to Ore-R controls (D, ANOVA, Dunnett's multiple comparison, P=0.01), but did not differ among the transgenic mutant lines when compared to $n$-$syb^{WT}$ (ANOVA, P>0.05).
Figure 32: Lifespan

Reduced lifespan observed in hypomorphic and RNAi lines was restored in the transgenic hypomorphs. Among the transgenic hypomorphs, \( n\text{-}syb^{AA} \) and \( n\text{-}syb^{L24} \), showed significantly longer life spans than \( n\text{-}syb^{WT} \) (ANOVA, Dunnett's multiple comparison, \( P<0.001 \)).
Morphology

We did not expect that the morphology of the neuromuscular junction would be altered by the \( n\)-syb transgenes because previous studies have found no effect of reduced levels of \( n\)-syb expression on NMJ development (Deitcher et al., 1998; Stewart et al., 2000). This was the case when comparing the overall length of the NMJ between control, hypomorph, wild-type transgenics or mutant transgenics in the hypomorphic background (Figure 33A, ANOVA, \( P>0.05 \)). Also, no difference was observed in the number of branches (Figure 33B, ANOVA, \( P>0.05 \)). However, we found a modest increase in the number of boutons observed in \( n\)-syb\textsuperscript{L6} and \( n\)-syb\textsuperscript{L24} transgenic mutants when compared to \textit{Ore-R} (Figure 33C, ANOVA, Dunnett's multiple comparison, \( P<0.001 \)), but no significant difference was observed when comparing the mutant to the \( n\)-syb\textsuperscript{WT} transgenic line.

Synaptic physiology

We next assessed the effect of the \( n\)-syb mutations on synaptic physiology. Our measurements of frequency and amplitude of spontaneous fusion events revealed no significant difference between control, hypomorphs and transgenics in the hypomorphic background (Figure 34, ANOVA, \( P>0.05 \)).

We analyzed evoked synaptic responses by stimulating the motor nerve and measuring the excitatory junctional potentials (Figure 35). As we expected the hypomorphic allele had smaller EJPs compared to the \textit{Ore-R} control (12.2±2.0mV vs. 28.6±1.2). The wild-type \( n\)-syb transgene substantially restored synaptic function to the hypomorph; we found EJPs of 28.9±1.3mV in \( n\)-syb\textsuperscript{WT} larvae, almost identical to that of \textit{Ore-R}. The addition of one tryptophan residue (\( n\)-syb\textsuperscript{WW} ) yielded EJPs that were similar to the wild-type transgenic (34.4±1.6mV), but larvae bearing a mutation of tryptophan to alanine (\( n\)-syb\textsuperscript{AA} ) had EJPs that were smaller than \( n\)-syb\textsuperscript{WT} (18.9±2.3mV). Insertion of a 6 residue linker in the linker region (\( n\)-syb\textsuperscript{L6} ) resulted in somewhat smaller EJPs (22.5±2.8 mV), but addition of a 24 residue linker (\( n\)-syb\textsuperscript{L24} ) did not seem to have an effect of synaptic transmission. EJPs in these larvae were 27.8±1.8mV, similar to \( n\)-syb\textsuperscript{WT}. Therefore a significant decrease in the amplitude of EJPs resulting from evoked neurotransmitter release was observed for \( n\)-syb\textsuperscript{AA} and \( n\)-syb\textsuperscript{L6}.
when compared to \( n\text{-syb}^{WT} \) (ANOVA, Dunnett’s multiple comparison test, \( P<0.05 \)). These results are in agreement with Maximov et al., (2009), who demonstrated a decrease in evoked release in hippocampal neurons with tryptophan to alanine mutations but differ somewhat from Deak et al., (2006), who demonstrated abolishment of evoked release with a 12 and 24 residue flexible insert.

We next sought to challenge the neurons with a higher rate of synaptic activity. Preparations were simulated at 10Hz for 5min to investigate possible effects of mutations in the juxtamembrane region of \( n\text{-syb} \) on the readily releasable or recycling pool of vesicles (Figure 36). A typical profile for wild-type lines stimulated at 10Hz is an initial depression of EJP amplitude for the first 6 seconds to approximately 90% of the pre-train amplitude, followed by potentiation to approximately 110% at about 2 minutes which is then maintained for the duration of the train (Seabrooke et al., 2010; Kisiel et al., 2011). The hypomorphic allele, which has a small initial output, showed an initial potentiation during the first 20 seconds, peaking at approximately 160% of the pre-train amplitude, and then maintained approximately 150% pre-train amplitude for the duration of the train (Figure 36A). The response of \( n\text{-syb}^{WT} \) in the hypomorphic background to 10Hz stimulation resembled the control, \( Ore-R \). The addition of one tryptophan (\( n\text{-syb}^{WW} \)), expressed in the hypomorphic background, was similar to the wild-type transgenic with initial depression, but with a faster return to pre-stimulus level (15s) followed by depression after approximately 2 minutes. Mutation of tryptophan to alanine (\( n\text{-syb}^{AA} \)) resulted in a profile similar to the wild-type transgenic, with initial depression followed by potentiation (Figure 36B). Addition of a 6 amino acid linker (\( n\text{-syb}^{L6} \)) resulted in a 10Hz stimulation profile similar to the wild-type transgenic with initial depression, but amplitudes never fully returned to pre stimulus levels and remained at approximately 80% pre-stimulus amplitude. Finally, the 24 amino acid flexible insert (\( n\text{-syb}^{L24} \)) resulted in a 10Hz stimulation profile similar to \( n\text{-syb}^{L6} \), but with initial depression occurring more slowly (Figure 36C).

Close inspection of the first 10 stimuli of the 10Hz trains can reveal an effect on the readily releasable pool of vesicles (Figure 36D-F). Both the \( n\text{-syb}^{WW} \) and \( n\text{-syb}^{AA} \) mutants, in the hypomorphic background, showed a similar rate of depression to \( n\text{-syb}^{WT} \), indicating no affect on the readily releasable pool. However, the addition of linkers to the juxtamembrane
region ($n$-syb$^{L6}$ and $n$-syb$^{L24}$) did result in greater initial depression in the first few stimuli. This may indicate a reduced number of vesicles in the readily releasable pool. The effect of reduced $n$-syb expression on synaptic transmission, in the hypomorphic or in $n$-syb-RNAi larvae, tested with 10Hz stimulation was found to be fundamentally different from wild-type or transgenic hypomorphs. Here, initial release was low, but quickly increased during the train, perhaps due to a buildup of residual calcium. Therefore mutation of tryptophan in the juxtamembrane region of $n$-syb did not affect the readily releasable pool, but insertion of flexible linkers did.

EJPs stimulated in a wild-type preparation tend to be well synchronized with respect to the stimulus used to evoked them where the time to peak amplitude is consistent on a pulse-by-pulse basis. Disruption to membrane fusion therefore, may be observed as a loss of synchronicity in time to EJP peak amplitude. We therefore measured the time of peak amplitude for each EJP in a series of 16 stimuli and calculated the variance of the response. For Ore-R larvae we found that the time to EJP peak occurred within a range of 1.8±0.2ms and had a variance of 0.27±0.06 at 0.1Hz stimulation. For 10Hz stimulation we observed a range of 1.9±0.2ms and a variance of 0.29±0.04 (Figure 37).

This consistency in time to peak was lost in the $n$-syb hypomorphic and $n$-syb-RNAi larvae, which showed a range of 3.9±0.5ms and 8.0±0.6ms respectively and corresponding variances of 1.4±0.4 and 6.6±1.0 for 0.1Hz stimulation and an even greater effect with 10Hz stimulation. Expression of $n$-syb$^{WT}$ in the hypomorphic background restored normal timing for synaptic transmission. The range was 1.9±0.2ms and had a variance of 0.33±0.07 for 0.1Hz stimulation and a range of 2.4±0.3ms and a variance of 0.42±0.12 for 10Hz stimulation (Figure 37).

$n$-syb$^{WW}$ synapses also showed temporally consistent EJPs, similar to that of Ore-R and $n$-syb$^{WT}$. However mutation of tryptophan to alanine, $n$-syb$^{AA}$, demonstrated an effect on the timing of synaptic transmission. Time to peak with 0.1Hz stimulation had a range of 2.6±0.4ms, with a variance of 0.67±0.23, while time to peak during 10Hz stimulation ranged by 4.5±1.0ms with a variance of 1.73±0.64. Therefore, increasing stimulation frequency to 10Hz did reveal a significant difference in variance of time to peak for transgenics in the hypomorphic background (P=0.03, ANOVA, Dunnett's multiple comparison test compared to
The insertion of flexible linkers into the juxtamembrane region did not have a significant effect on the timing of synaptic transmission in this system. In summary, elimination of tryptophan was observed to increase the variance in time to peak indicating a decrease in the synchronicity of release.

The quantal content of evoked release was measured using the method of failures. We found that quantal content was reduced in the hypomorph and n-syb-RNAi compared to Ore-R. This reduction was restored with expression of the wild-type transgene, n-syb\(^{WT}\) (Figure 38). Addition of one tryptophan residue to the juxtamembrane region, n-syb\(^{WW}\), demonstrated a quantal content similar to Ore-R and n-syb\(^{WT}\) but mutation of tryptophan to alanine, n-syb\(^{AA}\), demonstrated a significant reduction in quantal content. The insertion of flexible linkers into the juxtamembrane region, n-syb\(^{L6}\) and n-syb\(^{L24}\), also demonstrated significantly reduced quantal content (P<0.001, ANOVA, Dunnett's multiple comparison to n-syb\(^{WT}\)). Therefore, elimination of tryptophan or insertion of flexible linkers in the juxtamembrane region reduced quantal content.
Figure 33: Morphology

Morphological analysis of 3rd instar larval neuromuscular junction (NMJ) 6/7. Neither overall NMJ length (A), nor number of branches (B) differed significantly between groups (ANOVA, P>0.05). However, a significant increase in the number of boutons was observed in n-syb^{L6} and n-syb^{L24} compared to Ore-R (C, ANOVA, Dunnett's multiple comparison, P<0.001), but no significant difference was observed among the transgenic hypomorphs. The width of muscle 6 was found to be slightly larger in n-syb^{A4} compared to the wild-type transgenic in the hypomorphic background (D, ANOVA, Dunnett's multiple comparison, P=0.001). n=12.
Figure 34: Miniature Potentials

Spontaneous miniature excitatory junctional potentials (mEJPs) measured from muscle 6 of 3rd instar larval preparations using a sharp electrode (n = 10 to 15). Neither the amplitude (A), nor the frequency (B) of mEJPs differed significantly (ANOVA, P>0.05).
A. Sample Evoked Junctional Potentials

Ore-R

Hypomorph

n-syb^{WT}

n-syb^{RNAi}

n-syb^{WW}

n-syb^{AA}

n-syb^{L6}

n-syb^{L24}
B. Average Evoked Junctional Potentials

Figure 35: Evoked Junctional Potentials

Evoked excitatory junctional potentials (EJPs) measured from muscle 6 of 3\textsuperscript{rd} instar larval preparations using a sharp electrode (n = 10 to 15). 16 representative traces resulting from one cell for each line are plotted (A) (Scale 10mV X 10ms). The decrease in EJP amplitude observed in the hypomorph was restored to control (\textit{Ore-R}) levels with expression of \textit{n-syb\textsuperscript{WT}} in the hypomorphic background (B). Among the transgenic hypomorphs, a significant decrease in EJP amplitude was observed in \textit{n-syb\textsuperscript{AA}} and \textit{n-syb\textsuperscript{L6}} (ANOVA, Dunnett's multiple comparison test, P<0.05).
A. 10Hz stimulation: control lines

B. 10Hz stimulation: tryptophan mutants

C. 10Hz stimulation: linker mutants
D. First 10 stimuli at 10Hz: control lines

E. First 10 stimuli at 10 Hz: tryptophan mutants

F. First 10 stimuli at 10 Hz: linker mutants
G. Representative Traces

Ore-R

Hypomorph

n-syb$^{WT}$

n-syb-RNAi

n-syb$^{WW}$

n-syb$^{AA}$

n-syb$^{L6}$

n-syb$^{L24}$
Figure 36: 10Hz Stimulation

Evoked excitatory junctional potentials (EJPs) stimulated 16X0.1Hz, 3000X10Hz, 16X0.1Hz and normalized to the average amplitude for the first 16 stimuli (n=5 to 7). The typical wild-type profile of initial depression followed by sustained potentiation is contrasted by the hypomorph which demonstrated marked potentiation to approximately 160% pre stimulus level (A). Expression of \( n\text{-syb}^{WT} \) in the hypomorphic background resulted in a response to 10Hz stimulation similar to the control. The tryptophan mutant hypomorphs also produced similar stimulation profiles to the wild-type transgenic, but with less initial depression for \( n\text{-syb}^{WW} \) and more for the \( n\text{-syb}^{AA} \) (B). The flexible linker mutant hypomorphs showed greater depression than the wild-type transgenic and were unable to sustain release at pre stimulus levels for the duration of the train (C). The first 10 stimuli at 10Hz reveals a similar response for the wild-type transgenic compared to \( Ore-R \) (D), and both tryptophan mutant hypomorphs to the wild-type transgenic control (E). The flexible linker mutant hypomorphs however demonstrated greater depression in these initial stimuli compared to the transgenic control (F). Lines with reduced \( n\text{-syb} \) expression, the hypomorph and \( n\text{-syb}-RNAi \), demonstrate potentiation over the first 10 stimuli (E). The first 16 traces resulting from stimulation of one representative cell at 10Hz are plotted (G) (Scale 10mV X 10ms).
A. Average Range Between Time to Peak for Each Cell

B. Variance in Time to Peak

Figure 37: Synchronicity, variance in time to peak

Synchronicity of excitatory junctional potential (EJP) peak for each set of 16 stimuli per cell at 0.1 and 10Hz (n = 10 to 15). Average range between slowest and fasted EJP peak for each set of stimuli (A), and average variance of each set (B) are plotted. Compared to Ore-R control, an increase in the range and variance of peak time was observed for the hypomorphic and n-syb-RNAi lines which was restored to control levels with expression of the wild-type transgenic, n-syb<sup>WT</sup>. Among the transgenic hypomorphs, a significant increase in variance of time peak at 10Hz stimulation was observed for n-syb<sup>AA</sup> (P=0.03, ANOVA, Dunnett's multiple comparison test).
Quantal content determined via the method of failures by stimulating preparations 500X at 1Hz in 0.2mM CaCl$_2$ (n=6). Quantal content was reduced in the hypomorphic and $n$-syb-RNAi lines compared to Ore-R. This reduction was restored with expression of the wild-type transgene, $n$-syb\textsuperscript{WT}. Within the transgenic hypomorphs, $n$-syb$^{AA}$, $n$-syb$^{L6}$ and $n$-syb$^{L24}$ demonstrated reduced quantal content while $n$-syb\textsuperscript{WW} did not (P<0.001, ANOVA, Dunnett's multiple comparison).

**Figure 38: Quantal content (method of failures)**

Quantal content determined via the method of failures by stimulating preparations 500X at 1Hz in 0.2mM CaCl$_2$ (n=6). Quantal content was reduced in the hypomorphic and $n$-syb-RNAi lines compared to Ore-R. This reduction was restored with expression of the wild-type transgene, $n$-syb\textsuperscript{WT}. Within the transgenic hypomorphs, $n$-syb$^{AA}$, $n$-syb$^{L6}$ and $n$-syb$^{L24}$ demonstrated reduced quantal content while $n$-syb\textsuperscript{WW} did not (P<0.001, ANOVA, Dunnett's multiple comparison).
Discussion

A functional role for the juxtamembrane region of n-Syb in neurotransmitter vesicle fusion was confirmed using transgenic expression of wild-type and mutant forms of n-syb in a hypomorphic background (n-syb<sup>ΔF33B</sup>/n-syb<sup>ΔF33B</sup>). Mutation of the conserved interfacial tryptophan residue to alanine (n-syb<sup>AA</sup>) reduced the amplitude, synchronicity and quantal content of evoked release whereas addition of a tryptophan residue (n-syb<sup>WW</sup>) had relatively little effect and synaptic responses. n-syb<sup>WW</sup> resembled the wild-type transgenic (n-syb<sup>WT</sup>) in all tests performed. Addition of a flexible 6 amino acid segment to the juxtamembrane region (n-syb<sup>L6</sup>) reduced evoked release and decreased quantal content; while a 24 amino acid segment (n-syb<sup>L24</sup>) resulted in normal evoked release, but with decreased quantal content when measured in low calcium. This represents the first demonstration of mutations to a VAMP, expressed in a living animal model, where full electrophysiological and behavioural characterization could be performed.

The current results confirm and extend those reported previously in the literature with tryptophan 89 and tryptophan 90 to alanine mutations in VAMP2: Borisovska et al., 2012, demonstrated reduced vesicle priming in chromaffin culture cells, Maximov et al., 2009, demonstrated decreased evoked release from hippocampal neural culture cells and Quetglas et al., 2002 demonstrated reduced secretion from PC12 cells. However, two other studies reported somewhat different results using the liposome fusion assay with an increase in fusion (Kweon et al., 2003), or no effect (Siddiqui et al., 2007) using VAMP2 with tryptophan 89 and tryptophan 90 to serine mutations. These results are contrasted by an observed decrease in the exocytotic burst phase from chromaffin cells expressing tryptophan 89 and tryptophan 90 to serine mutations (Borisovska et al., 2012). It should not be assumed that the substitution of tryptophan for alanine or serine would have the same effect at the membrane where hydrophilic serine is intended to expose the membrane proximal region whereas hydrophobic alanine is intended to replace tryptophan but not perturb the protein's membrane association. Indeed, our recent biophysical study of n-syb with tryptophan 89 and leucine 90 to alanine mutations demonstrated deeper insertion and reduced anchoring in a DPC micelle (Al-Abdul-Wahid et al., 2012). Therefore, it would appear likely that the physiological effect of reduced
evoked neurotransmitter release may result from decreased anchoring of VAMP to the vesicular plasma membrane.

While no effect was observed on miniature potentials, this data is in agreement with Stewart et al., (2000), who demonstrated no difference in the frequency or amplitude of mEJCs for Ore-R control and hypomorphic larvae. However, Maximov et al., 2009, demonstrated an increased frequency of miniature potentials recorded from hippocampal neuronal culture cells with tryptophan to alanine mutations (VAMP2, tryptophan 89 and tryptophan 90 to alanine). While in a similar preparation, Deak et al., 2006, demonstrated normal frequency miniature potentials with a 12 residue flexible insert in VAMP2 but an elimination of miniature potentials with a 24 residue flexible insert. Differences observed may be because the low levels of endogenous n-Syb produced in the hypomorph are sufficient to conduct spontaneous fusion. However there are fundamental differences between the Drosophila neuromuscular synapse and the mouse hippocampal synapse that complicate direct comparison of these results.

Tryptophan residues are common in transmembrane proteins near the end of transmembrane regions (Wimley and White, 1996) and are thought to determine the precise positioning of membrane proteins (Ridder et al., 2000; Killian and von Heijne, 2000). But beyond simple localization, tryptophan residues have been hypothesized to play a direct role in facilitating membrane fusion through anchoring, lipid mixing (Sutton et al., 1998; Hu et al., 2002; Jahn and Sudhof, 1999), and by imparting an angle to the transmembrane region (Bowen and Brunger, 2006). Interestingly, viral fusion proteins, which must overcome the same fundamental biophysical challenges as SNARE proteins, often have conserved tryptophan residues localized at the membrane interface. Indeed, mutation of tryptophan residues in fusion proteins of Herpes simplex virus, HIV, Influenza and Ebola have been found to inhibit membrane fusion (Galdiero et al., 2008; Salzwedel et al., 1999; Lai and Tamm, 2007; Freitas et al., 2007).

Therefore it was not surprising that transgenic expression of n-syb^AA, displayed reduced evoked fusion and decreased quantal content, while n-syb^WW resembled the wild-type. We did not observed an increased spontaneous release frequency similar to that observed by Maximov et al., 2009. This may be explained because our transgenes are expressed in the
hypomorphic background, which produces low levels of endogenous n-Syb or a difference in the model employed. No evidence for a difference in vesicle docking or mobilization was found with 10Hz stimulation indicating that the electrophysiological phenotype observed resulted during evoked release and was not a secondary artifact of a trafficking problem. Furthermore, a deficit to synaptic transmission in n-syb\textsuperscript{AA} mutants was demonstrated with decreased synchronicity of fusion and reduced quantal content. It must be noted that the hypomorph alone also showed reduced evoked release, decrease synchronicity and reduced quantal content however the magnitude of these effects were greater than that observed for n-syb\textsuperscript{AA} indicating that the mutant transgene is functioning in synaptic transmission, but just not as well as n-syb\textsuperscript{WW} or n-syb\textsuperscript{WT}. Furthermore, a profound difference was observed in the response to 10Hz stimulation between n-syb\textsuperscript{AA} and the hypomorph perhaps indicating a reduction in vesicle mobilization in the hypomorph due to its reduced level of n-syb expression.

Interestingly, even with a significant reduction in evoked release, observed in transgenic n-syb\textsuperscript{AA} expression, climbing ability and development were similar, or longer in the case of lifespan, to Ore-R and n-syb\textsuperscript{WT}. This contrasts sharply with the hypomorph which was unable to climb and only survived for a few days as adults. Therefore, although n-syb\textsuperscript{AA} was shown to inhibit synaptic transmission in our tests, animals expressing the mutation were able to cope and survive. It would be interesting to determine if the reduction in evoked release observed in the larval preparation could cause more subtle phenotypes during the lifetime of the fly, such as impairment of learning and memory or social behaviours.

The short distance between the SNARE and transmembrane regions may be important to bring opposing membranes into close apposition to facilitate membrane fusion. However, the addition of flexible inserts between arginine 86 and lysine 87 in n-syb produced mixed results. Transgenic expression of n-syb\textsuperscript{L6} resulted in reduced evoked release but transgenic expression of n-syb\textsuperscript{L24} unexpectedly showed normal amplitude EJPs. Previous experiments analyzing vesicle fusion from hippocampal neuronal culture cells with 12 and 24 amino acid inserts also between arginine 86 and lysine 87 eliminated evoked release (Deak et al., 2006). In addition, a trend of increased inhibition of fusion with increased insertion length between leucine 93 and methionine 95 was observed using liposomes (McNew et al., 1999) and
chromaffin cells (Kesavan et al., 2007). With 10 Hz stimulation, the two linker mutants were unable to maintain evoked release at pre-stimulus levels which may indicate some effect on vesicle cycling. Flexible linkers inserted in VAMP2 have been demonstrated to have an effect on the recycling pool of chromaffin cells (Kesavan et al., 2007).

Therefore, transgenic expression of n-sybL6 fits the hypothesis and previous data, with reduced evoked transmission, decreased quantal content and an effect on the readily releasable and recycling pools. While n-sybL24 larvae showed normal evoked transmission, it did show reduced quantal content in low calcium and an effect on the readily releasable and recycling pools. This mutation was expected to eliminate evoked transmission and potentially function in a dominant negative fashion (Kesavan et al., 2007), but in our experiments, synaptic physiology was not drastically affected. One explanation would be that Syntaxin acted to stabilize formation of the coiled-coil from SNARE motif assembly, through the juxtamembrane and transmembrane regions (Stein et al., 2009). The 24 amino acid flexible linker could be long enough to simply fold out of the way and have no effect at all. This could be tested with expression of a similar insert in Syntaxin as demonstrated by McNew et al., (1999) in the liposome fusion assay. Therefore, although the observed normal amplitude EJPs resulting from n-sybL24 expression in the hypomorphic background lends no evidence to support a requirement for a short rigid segment between the SNARE and transmembrane regions for membrane fusion, it also does not disprove it. Furthermore, inhibition to evoked synaptic transmission was observed in n-sybL6 and both mutants showed detriments to synaptic physiology.

In the case of both linker mutants, time course for development, lifespan and neuromuscular junction morphology were unaffected by the structural change. However climbing ability in the n-sybL24 mutant was somewhat reduced, where a smaller percentage of flies completed the task, they were slower in the time taken to reach the top and demonstrated decreased overall height achieved. This may indicate that some of the physiological differences observed in the larvae may ultimately foreshadow a subtle effect on motor ability in the adult fly.

The phenotypes observed in this study predominantly result from transgenic expression of wild-type and mutant n-syb and are not simply an artifact of the hypomorphic
phenotype. The hypomorph demonstrated reduced EJPs, decreased quantal content, decreased synchronicity, potentiation with 10Hz stimulation, reduced lifespan and severe loss of motor coordination in the adult. These results were replicated with another model of reduced n-syb expression: UAS-n-syb-RNAi driven by the pan neural promoter ELAV-Gal4. It is expected that the low level of endogenous n-Syb could attenuate effects resulting from transgenic expression of mutant constructs, particularly with miniature potentials. However, none of the transgenic lines demonstrate the potentiation observed with 10Hz stimulation of the hypomorph and all of the lines show marked improvement in motor ability and lifespan over the hypomorph. Additional lines representing each mutation, arising from different P-element insertions, were tested and similar results observed (data not shown). If a particular transgenic construct expressed a mutation which was unable to facilitate membrane fusion, but did not cause a dominant negative effect, then one might expect no difference from the hypomorphic phenotype and conclusions would be difficult, but this was not the case with our mutants. Ideally, mutants would have been expressed in a null background and genomic constructs included the endogenous promoter to fulfill this goal. And indeed, protein expression and correct membrane localization were observed in Drosophila S2 cells, however wild-type and mutant constructs were unable to rescue the null mutant and thus the hypomorph was used. This important finding directs future studies attempting genomic rescue to include large regions of DNA flanking the gene to increase the chances of including all regulatory regions of the gene.

In conclusion, analysis of transgenic Drosophila expressing wild-type and mutant n-syb demonstrate that the juxtamembrane region plays a functional role in membrane fusion and is not simply a linker between the SNARE and transmembrane regions. Substitution of the interfacial tryptophan residue significantly impairs synaptic physiology, as does insertion of a short flexible linker between the SNARE and transmembrane regions.
Chapter 5  General Discussion

General findings

Results of this genetic, physiological and structural study clearly demonstrate that neuronal-Synaptobrevin (n-Syb) is a protein necessary for the fusion of neurotransmitter containing vesicles in neurons of Drosophila melanogaster. This work represents the first correlated structural, genetic and physiological study, from an atomic to a behavioural level, of a synaptically important molecule. And, because of the highly conserved nature of VAMPs, the results of this study are directly applicable to all animals that utilize Ca\textsuperscript{2+} evoked neurotransmitter release and have general application in understanding the fundamental mechanisms behind broad membrane fusion events such as vesicle fusion during intracellular trafficking, exocytosis and viral fusion.

This combined approach in investigating the role of the juxtamembrane region of n-Syb in neurotransmitter vesicle fusion allowed for the testing of hypotheses ranging from the basic structural, atomic level to how these structural changes affect synaptic physiology. This work represents the first example where these types of specific structural questions have been assessed in actual living animals. This study also provides insight into the benefits and challenges associated with a comparative approach to studying SNARE proteins.

While previous studies have pointed to a role for membrane proximal tryptophan residues in positioning and anchoring membrane proteins in general (Wimley & White, 1996) and VAMPs specifically (Kweon et al., 2003a), biophysical analysis of tryptophan mutations in VAMPs have not been performed prior to this study. The hypothesis that the conserved tryptophan residue in the linker region of n-Syb anchored and positioned the molecule in the membrane was tested with \textsuperscript{19}F NMR in DPC micelles. From 1D spectra collected at different temperatures, it was found that the addition of one tryptophan residue increased anchoring to the micelle. Depth of insertion was tested by observing the oxygen induced contact shift and solvent induced isotope shift. In both cases, mutation of tryptophan to alanine caused the molecule to insert deeper into the micelle, while addition of one tryptophan caused the
molecule to be more exposed to the solvent. The observed reduction in anchoring and increase in depth of insertion with tryptophan mutated to alanine supported this hypothesis and represented the first atomic level structural study of this type of mutation in the VAMP/Synaptobrevin family.

Now with direct evidence that membrane proximal tryptophan residues are involved in anchoring n-Syb to the membrane (Al-Abdul Wahid et al., 2012) and reports that these residues play a role in exocytosis from PC12 cells (Quetglas et al., 2002), in liposome fusion (Kweon et al., 2003a), in secretion from cultured chromaffin cells (Borisovska et al., 2012) and in neurotransmitter release from cultured hippocampal neurons (Maximov et al., 2009), a reverse genetic strategy was employed to tackle this question in the Drosophila model. First, if tryptophan played a functional role in membrane fusion then mutation of tryptophan to alanine would inhibit neuronal transmission. Second, if the linker region of n-Syb must be short and rigid for the transduction of force from SNARE complex assembly to the membrane to facilitate fusion, then insertion of flexible linkers would inhibit neuronal transmission. Here, specific mutations to n-syb could be made, inserted into the genome using P-element transformation, and expressed transgenically in a variety of backgrounds. The difficulties associated with this approach include the laborious nature of determining the position of each insert and genetic manipulation to produce flies with the desired compliment of genes to test transgenic expression. Specific to this project, challenges resulted from the fact that a strategy to include the endogenous promoter was not successful in rescue of the n-syb null, therefore the transgenes were tested in an n-syb hypomorphic background. The benefits of this model exceeded the challenges. First, detailed synaptic analysis using electrophysiological techniques was performed at the well characterized 3rd instar larval neuromuscular junction. Second, the fact that transgenics in the hypomorphic background survived to the adult stage allowed development, lifespan, morphology and climbing ability to be measured. None of the other models provide an animal with which to perform comprehensive experiments upon.

Five types of transgenic flies were produced: wild-type (n-syb$^{WT}$), mutation of tryptophan to alanine (n-syb$^{AA}$), addition of one tryptophan (n-syb$^{WW}$), insertion of a six amino acid flexible linker (n-syb$L6$) and insertion of a 24 amino acid flexible linker (n-syb$L24$). Transgenic lines readily produced wild-type and mutant transgenic n-Syb which was found to
localize to head extracts indicating neural expression. The hypomorph produced adult flies with severe motor inhibition and reduced lifespan which was restored to wild-type levels by expression of wild-type transgenic $n$-syb. While spontaneous fusion events were not affected by the mutations, evoked potentials were found to decrease in $n$-syb$^{AA}$ and $n$-syb$^{L6}$ mutants. In a reduced concentration of CaCl$_2$, quantal content was observed to decrease in all mutants except for $n$-syb$^{WW}$. These results are mostly in agreement with those obtained in other systems.

The study of fusion using SNARE proteins reconstituted into liposomes has provided fundamental understanding of SNARE function including the demonstration that three SNARE proteins alone are capable of inducing membrane fusion (Weber et al., 1998). The ability to reduce the system to the minimal number of molecular participants is the true strength of this model however, rates of fusion are not equivalent to in vivo synapses, perhaps due to missing molecular participants. Therefore, arguments will always arise about the molecular composition of the fusion apparatus, the concentration of expressed proteins and the size and phospholipid composition of the liposomes in comparison to an endogenous scenario. With respect to tryptophan mutation, data from this model conflict where an increase in fusion between liposomes was observed (Kweon et al., 2003b; tryptophan to serine mutation), or no change in fusion was observed (Siddiqui et al., 2007; tryptophan to alanine mutation). In contrast, the Drosophila model clearly demonstrated a deleterious effect of tryptophan to alanine mutation on fusion. Insertion of flexible linkers into VAMP and/or Syntaxin inhibited fusion in the liposome model to a greater degree than that observed in the Drosophila synaptic preparation (McNew et al., 1999).

Cellular models used to measure vesicle fusion through secretion such as PC12 (Quetglas et al., 2002) cells and Chromaffin cells (Kesavan et al., 2007; Borisovska et al., 2012) have been valuable in testing hypotheses related to SNARE protein mediated fusion. Both Quetglas et al., (2002), and Borisovska et al., (2012), demonstrated a decrease in secretion with tryptophan to alanine mutation in agreement with the Drosophila model. However, Both Kesavan et al., (2007) and Borisovska et al., (2012), observed a greater reduction in secretion with the introduction of flexible linkers in the juxtamembrane region than that observed in the Drosophila model. In Chromaffin cells, secretion is generally
measured using carbon fibre amperometry, where vesicle fusion leads to an increase in capacitance. Secretion can be stimulated by flash photolysis, releasing Ca\(^{2+}\), causing a coordinated burst in secretion. Measurements are sensitive and a great deal of information about the kinetics of membrane fusion can be gained. However, these cellular preparations are not synapses and therefore hypotheses regarding the effect of structural mutation of SNARE proteins on Ca\(^{2+}\) evoked transmitter release must also be tested using synaptic models.

One such model was employed by Deak \textit{et al.}, (2006) and Maximov \textit{et al.}, (2009), to study the effect of mutation to VAMP and other components of the release apparatus on Ca\(^{2+}\) evoked neurotransmitter fusion. Mice were crossed to produce embryos null for mammalian neural VAMPs, which is lethal. But, before death, the embryos were dissected and cortical or hippocampal neurons plated. These cultured neurons form both inhibitory and excitatory synapses. Expression of transgenic protein was induced using infection with lentivirus, encoded to produce the transgene being tested. Then, standard electrophysiological experiments could be performed on the neurons. Maximov \textit{et al.}, (2009), expressed VAMP with tryptophan to alanine mutations and observed a reduction in evoked fusion and an increase in spontaneous fusion. While these data fit well with those from the \textit{Drosophila} model, an increase in spontaneous fusion was not observed. As discussed in Chapter 4, this may have resulted from low level expression of endogenous n-Syb in the hypomorph, or from a fundamental difference between central and neuromuscular synapses. Deak \textit{et al.}, (2006), observed a more drastic effect of the insertion of flexible linkers into the juxtamembrane region of VAMP on neurotransmitter vesicle fusion than that observed in the \textit{Drosophila} model. Here a reduction in fusion was observed with the 6, but not the 24 residue linker. Compensatory alignment with Syntaxin may explain the relatively uninhibited level of neurotransmitter vesicle fusion with the 24 residue insert and further experimentation is necessary. While cultured hippocampal and cortical neurons represent a truly exceptional system for testing the effect of mutation to SNARE proteins on synaptic transmission, synapses are formed in an \textit{in vitro} environment. Also, expression of transgenic protein is not under normal endogenous control. Furthermore, the synapses represented in this model, while important, represent only a subset of the type of synapses found in the nervous system and testing hypotheses in other synaptic models is warranted.
Therefore transgenic expression under endogenous control in *Drosophila*, provides a highly valuable model with which to test hypotheses related to the effect of mutation of SNARE protein structure on Ca\textsuperscript{2+} evoked neurotransmitter fusion. The neuromuscular junction provides a synaptic model to contrast with those formed by cultured hippocampal and cortical cells. Most importantly, the animal's survival from embryo through larval, to adulthood provides an exceptional opportunity for a diverse array of experiments on the effect of SNARE protein mutation in a living animal model.

**Future Opportunities**

While biophysical analysis of n-Syb tryptophan mutants in a DPC micelle using \textsuperscript{19}F NMR spectroscopy did demonstrate an effect of tryptophan on anchoring n-Syb to the membrane, further analysis could prove valuable. The first step would be to assign all peaks in the HSQC spectra by producing \textsuperscript{13}C and \textsuperscript{15}N labeled proteins. This would allow for a more detailed analysis on the effect of tryptophan mutation on individual residues in addition to the \textsuperscript{19}F tagged aromatic residues. Next, the question of why mammalian VAMP2 has one additional tryptophan residue compared to *Drosophila* n-Syb could be tackled. I hypothesize that perhaps fundamental differences in mammalian and insect membranes may result in differences in the amino acid sequences of neural VAMPs. While mammalian membranes contain a high phosphatidylcholine content with a substantial amount of the negatively charged phospholipid phosphatidylycerine, Dipteran membranes are predominantly composed of phosphatidylethanolamine with very little phosphatidylycerine (Luukkonen *et al*., 1973). Testing membrane anchoring of constructs representing the mammalian or *Drosophila* VAMP in bilayer models (Diller *et al*., 2009) composed of phospholipids in proportions similar to mammalian or *Drosophila* membranes would be warranted to determine if the sequence differences have a functional role in anchoring.

Besides tryptophan, other interesting residues in the linker region of *n-syb* could be tested using the *in vitro* membrane models and *in vivo* physiological models employed in the aforementioned studies. The sequence (83-KLKRKFWLQNLK-94) contains several conserved lysine and arginine residues which may function in membrane anchoring (Strandberg *et al*., 2002) and therefore membrane fusion. Kim *et al*., (1999), demonstrated
that four lysine residues: lysine 83, lysine 87, lysine 91 & lysine 94 form the binding signal for VAMP2 membrane integration in the rat (*Rattus norvegicus*) and that the asparagine at position 92 was also important for membrane integration. Perhaps these residues could play a role in membrane anchoring and fusion after protein integration into the membrane. Maximov *et al.*, (2009) demonstrated a reduction in evoked release from hippocampal culture neurons expressing lysine 85 and arginine 86 to alanine or arginine 86 and lysine 87 to alanine mutations. Also of interest is the conserved aromatic residue at position 88 which is phenylalanine in n-Syb and tyrosine in VAMP2. Because, in our model, tryptophan 89 and leucine 90 mutation to alanine, expressed in the hypomorphic background, was mostly tolerated, introducing an additional mutation of phenylalanine 88 to alanine would be valuable. It is hypothesized that removal of all aromatic residues from the juxtamembrane region of n-Syb would further reduce membrane anchoring and further inhibit neurotransmitter vesicle fusion. The systematic reduction of aromatic and lysine residues known to, or suspected to, play a role in membrane anchoring would be well worth testing in this system to fully characterize the link between membrane anchoring and SNARE mediated neurotransmitter vesicle fusion.

Further examination of residues in the juxtamembrane region of n-Syb demonstrated to interact with other proteins should be targeted for mutational analysis and tested using *in vitro* and *in vivo* models for molecular interaction and physiological effects respectively. Residue 88 (tyrosine in VAMP2) was demonstrated to interact with adjacent tyrosine and glutamine residues in the juxtamembrane region of Syntaxin1A in the fully assembled *cis* SNARE complex. In addition, residue 92, which is asparagine in both VAMP2 and n-Syb, was demonstrated to interact with an alanine and lysine residue in the Syntaxin juxtamembrane region (Stein *et al.*, 2009). The sequence of the juxtamembrane region of mammalian Syntaxin1A is identical to *Drosophila* Syntaxin1A (NH-KYQSKARRKK-COOH) indicating that results of this study are likely relevant to n-Syb. Therefore, mutation of these residues may affect VAMP/Syx assembly, independent of membrane anchoring, causing an effect on membrane fusion. While VAMP2 forms many molecular interactions with other proteins, such as the SNARE complex and complexin (Chen *et al.*, 2002), these interactions are not known to occur in the juxtamembrane domain. However, interactions with
complexin should be kept in mind when conducting mutational analysis of the juxtamembrane region of n-Syb to study membrane fusion.

Other regions of n-Syb may be important in interacting with the membrane to facilitate neurotransmitter vesicle fusion such as the transmembrane and intravesicular regions. Many residues in the transmembrane region are highly conserved amongst VAMPs (Figure 6) and interaction between several residues in VAMP2 have been observed with Syntaxin1A in the fully assembled SNARE complex (Stein et al., 2009). These include residues: 95 (methionine), 98 (isoleucine), 99 (leucine), 102 (isoleucine) and 106 (isoleucine in VAMP2 or valine in n-Syb). Mutational analysis of these residues which interact with Syntaxin and other residues which may interact with the membrane would be valuable in determining the role of the transmembrane region in SNARE mediated membrane fusion. The role of the extensive intravesicular domain, present in n-Syb, but absent in VAMP2 should also be investigated. An initial study would first simply involve deletion of the domain to determine possible integration, trafficking or physiological role for this region of the protein. If an effect was observed, then more detailed analysis on specific regions or residues in the intravesicular domain could be initiated.

While the effect of the insertion of flexible linkers in the juxtamembrane region of VAMPs has been tested in this work and other studies (McNew et al., 1999; Deak et al., 2006, Kesavan et al., 2007; Borisovska et al., 2012) further investigation would be valuable. First, the insertion of rigid linkers could be used to determine if increasing the distance, but not flexibility between the SNARE and transmembrane regions would have an effect on membrane fusion. Second, testing the insertion of linkers in the juxtamembrane region of Syntaxin and VAMP at the same time, as performed by McNew et al., (1999; liposome fusion assay), would be useful. Further study should be conducted in cellular models and with rigid linkers. Potential compensatory effects of Syntaxin stabilizing flexible inserts in n-Syb would be eliminated if both molecules possessed similar inserts.

While n-syb genomic constructs performed well in the hypomorphic background, the fact that rescue of the n-syb null was unsuccessful presents a valuable opportunity for further study. Transgenic protein was produced in Drosophila S2 cells and oriented correctly in the membrane. Yet transgenic expression did not replace the function of endogenous n-Syb in the
null mutant. This result may point to complex regulation of expression of \textit{n-syb} in the fly. Identifying and characterizing potential regions of the genome responsible for regulating \textit{n-syb} expression would provide insight into protein regulation in general and synaptic proteins specifically. SNARE proteins are expressed early and responsible for many processes during development beginning at fertilization and continuing through cell division, neurite outgrowth and synapse formation. Patterns of expression and regulation of expression of these proteins are not well understood and further investigation is warranted (Hepp and Langley, 2001).

Constructs, including different portions of the genome in the vicinity of \textit{n-syb}, could be associated with a reporter molecule such as GFP. Location and timing of expression of \textit{n-syb} could be tracked through development. Once regulatory sequences are identified, the genome could be searched to determine if similar regulatory regions have similar affects on other genes. Regulatory regions could then be engineered for use in transgenic constructs to fine tune expression.

While the level of transgenic \textit{n-syb} expression was not found to correlate to restoration of climbing ability when expressed in the hypomorphic background, level of endogenous \textit{n-syb} expression does have an obvious effect. With no expression, observed in the null (\textit{n-syb}^{AF33B}), development progresses to the embryo but is lethal beyond this point. Low level expression, observed in the hypomorph (\textit{n-syb}^{I4}), progresses through larval, pupal and to short lived and severely uncoordinated adults. But does the null, in a heterozygous form, produce normal levels of n-Syb? It was observed that less adult null heterozygotes were produced compared to their wild-type siblings implying some lethality in the null heterozygotes. While, upon gross inspection, the null heterozygotes appeared normal, further investigation including electrophysiological and behavioural analysis should be performed and level of \textit{n-syb} expression determined. If two good copies of \textit{n-syb} are necessary for normal development and behaviour it would have serious implications. Heterozygous null SNAP-25 mutants have deficits to synaptic function (Pozzi \textit{et al.}, 2008; Scullin \textit{et al.}, 2012). Mutations, such as single nucleotide polymorphisms, could occur and be maintained in a population in a heterozygous form. Such mutations to SNAP-25 have been shown to have negative effects on cognitive performance and neuromuscular disease in humans (Cagliani \textit{et al.}, 2012).
While transgenic expression of tryptophan and linker mutants in an \textit{n-syb} hypomorphic background displayed electrophysiological phenotypes, lifespan and climbing ability were relatively normal. This does not preclude more subtle phenotypes such as learning and memory deficits or neuromuscular deficits in older flies and thus should be investigated. It is surprising that mutations proven to be deleterious to synaptic transmission are tolerated in the adult fly. It would be valuable to investigate the mechanisms employed by the fly to compensate for these and other mutations. For example, the stimulus pattern in a motor behaviour such as walking could be determined in the wild-type fly and compared to mutants to determine if the fly changes its neural output to compensate. It would also be interesting to further explore the ultimate limitations to the mutant flies. For example, the linker mutants (\textit{n-syb}\textsuperscript{L6} and \textit{n-syb}\textsuperscript{L24}) showed some deficit in trafficking during 10Hz stimulation and \textit{n-syb}\textsuperscript{L24} specifically showed some detriment to climbing ability compared to the transgenic wild-type. The first step would be to employ more demanding stimulation protocols with longer trains of stimuli at higher frequencies. Next, the flies could be tested in a situation of forced activity such as flying or walking (Seelig \textit{et al.}, 2010) to determine if the mutants have reduced stamina compared to control flies.

Quantal content was measured using the method of failures in a low concentration of calcium. If the number of release sites is the same between mutants, then these results would represent the probability of fusion. Therefore, determining the number of release sites through antibody staining for active zones, and analysis using confocal or electron microscopy would be valuable in determining the release probability of the mutants tested in this study.

Conclusions

In conclusion, the conserved tryptophan residue in n-Syb, is important for anchoring and positioning the molecule in the membrane. Mutation of tryptophan to alanine inhibited evoked neural transmission and reduced quantal content, while the addition of one extra tryptophan did not. Therefore the highly conserved interfacial tryptophan residue in n-Syb does play a role in membrane fusion. Insertion of a short flexible linker in the juxtamembrane region also inhibited evoked synaptic transmission and reduced quantal content.
References


Appendices

Appendix 1: Additional Data characterizing n-Syb constructs for NMR analysis

The following figures were generated from analysis of constructs used in NMR analysis (Chapter 2) but were not included in (Al-Abdul-Wahid et al., 2012) and are thus not included in Chapter 2.
Figure 39: Helical wheele analysis of n-Syb peptides

The figure was made using the Zidovetzki lab helical wheel generator (rzlab.ucr.edu). Legend: hydrophilic circle, hydrophobic diamond, negative charge triangle, positive charge pentagon.
Figure 40: Purification of n-Syb peptides

Coomassie Blue (A) Ladder, Flow through, SDS wash, imidazole wash, elution 1, 2, 3, 5, 7, 11. Western Blot with anti-6-His (B) Ladder, Flow through, imidazole wash, elution 1, 2, 3, 4, 5, 6, 7.
Figure 41: CD spectroscopic analysis of n-Syb peptides

Control spectra from buffer (60mM sodium phosphate, 2mM sodium fluoride, 0.5% DPC, pH6.5) was subtracted from sample spectra. Concentration of samples (30 to 50μM) were determined via amino acid analysis with 3 replicates per sample (APTC, Toronto). All samples were diluted 15X before spectra were recorded. Spectra were transformed using the equation: (recorded value / (10*path length*concentration*number residues)). Units are: Mean molar ellipticity per residue (degrees·cm²·dmol⁻¹) vs. wavelength (nm).
Figure 42: $^1$H$^{15}$N HSQC spectra

Overlay of spectra resulting from n-Syb constructs from wild-type (black), addition of one tryptophan (blue; mutation of leucine 90 to tryptophan) and no tryptophan (red; mutation of tryptophan 89 and leucine 90 to alanine) (A). Expanded portions of the spectra demonstrate: the glycine backbone (B); asparagine and glutamine NH$_2$ groups (C); tryptophan indole group (D); peptide backbone N-H bonds (E).
Figure 43: $^{19}$F chemical shift of WT-n-Syb

Spectra resulting from biosynthetic labels: 4-fluorophenylalanine (phenylalanine 77, phenylalanine 88) at 293ºK (A), 310ºK (B) and 318ºK (C); 5-fluorotryptophan (tryptophan 89) at 293ºK (D), 310ºK (E) and 318ºK (F).
Figure 44: $^{19}$F chemical shift of WW-n-Syb

Spectra resulting from biosynthetic labels: 4-fluorophenylalanine (phenylalanine 77, phenylalanine 88) at 293ºK (A), 310ºK (B) and 318ºK (C); 5-fluorotryptophan (tryptophan 89, tryptophan 90) at 293ºK (D), 310ºK (E) and 318ºK (F).
Figure 45: $^{19}$F chemical shift of AA-n-Syb

Spectra resulting from biosynthetic labels: 4-fluorophenylalanine (phenylalanine 77, phenylalanine 88) at 293°K (A), 310°K (B) and 318°K (C); 5-fluorotryptophan (none) at 293°K (D), 310°K (E) and 318°K (F).
Figure 46: $^{19}$F chemical shift of F77A-n-Syb

Spectra resulting from biosynthetic labels: 4-fluorophenylalanine (phenylalanine 88) at 293°K (A), 310°K (B) and 318°K (C); 5-fluorotryptophan (tryptophan 89) at 293°K (D), 310°K (E) and 318°K (F).