IN VIVO FUNCTION AND INTERACTION OF SNARES, NSF AND SNAP DURING
PRESYNAPTIC VESICLE TRAFFICKING IN DROSOPHILA

A Dissertation in

Genetics

by

Wenhua Yu

© 2010 Wenhua Yu

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2010
The dissertation of Wenhua Yu was reviewed and approved* by the following:

Zhi-Chun Lai  
Professor of Biology, Biochemistry and Molecular Biology  
Chair of Committee

Douglas R. Cavener  
Professor of Biology

Aimin Liu  
Assistant Professor of Biology

Wendy Hanna-Rose  
Associate Professor of Biology, Biochemistry and Molecular Biology

Richard W. Ordway  
Associate Professor of Biology  
Dissertation Advisor  
Chair of Intercollege Genetics Graduate Program

*Signatures are on file in the Graduate School
ABSTRACT

Synaptic transmission is a fundamental aspect of nervous system function. At chemical synapses, neurotransmitter-filled synaptic vesicles fuse to the presynaptic plasma membrane at specific sites known as active zones and release neurotransmitters, which act on postsynaptic neurons. SNAREs, NSF and SNAP are core components of the synaptic vesicle fusion apparatus. Gaining insights into their in vivo functions and interactions has been the primary research goal of this dissertation project. Taking advantage of the powerful genetic model system of Drosophila, the project has combined genetic, biochemical and live imaging methods to reveal interesting insights into synaptic protein behavior and interactions at living synapses. This work extends our previous analysis of a temperature-sensitive (TS) paralytic NSF mutant, comatose, and extends previous models describing the spatial organization of SNAREs, NSF and SNAP proteins with respect to presynaptic active zones.

Specifically, biochemical analysis demonstrated for the first time that acute disruption of dNSF1 activity in comatose leads to accumulation of protein complexes containing dNSF1, dSNAP and SNAREs, suggesting that mutant dNSF1 is capable of associating in complexes with dSNAP and SNAREs, although its ability to disassemble them is impaired by the missense mutation located in the D1 domain. Moreover, dNSF1, dSNAP and t-SNAREs exhibit activity-dependent redistribution to Peri-Active Zone (PAZ) regions of the plasma membrane in comatose, suggesting the PAZ as the location where NSF-mediated disassembly of SNARE complexes would normally take place. Further, dSNAP exhibits a stable punctuate distribution pattern to the PAZ following its redistribution in comatose but is still mobile and can disassociate from the PAZ, suggesting dSNAP may bind and disassociate from dNSF1 and SNARE complexes independent of SNARE complex disassembly. Moreover, the apparent shift of this equilibrium towards dSNAP binding to the PAZ may reflect accumulation of dNSF1 and SNAREs, which leads to higher capacity of affinity for dSNAP binding at the PAZ. Unlike dSNAP, dNSF1 is fixed at the PAZ following its redistribution in comatose, suggesting that anchorage or immobilization of dNSF1 at the PAZ is mediated by synaptic components or signals other than the classic synaptic vesicle fusion apparatus (SNAREs and SNAP).

Live imaging approaches developed and adapted in our laboratory, including in vivo Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Resonance Energy Transfer (FRET) analysis, have been employed to monitor the behavior and interactions of synaptic proteins at living synapses. Particularly, efforts have been made to establish, optimize and compare two independent FRET methods within the model system. Notably, for the first time in any system, intermolecular FRET was observed between presynaptic proteins and defined activity-dependent protein interactions within living presynaptic boutons. Live imaging analysis has also generated other interesting insights with respect to in vivo orientation of proteins in assembly within complexes and assessments of protein mobility at native synapses. These imaging studies have provided important in vivo evidence from individual living presynaptic boutons which complements and extends previous biochemical analysis in developing molecular models of neurotransmitter release mechanisms.
# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................................ viii

LIST OF TABLES .................................................................................................................................. x

ACKNOWLEDGEMENTS ....................................................................................................................... xi

Chapter 1  Introduction ........................................................................................................................... 1

1.1 Chemical synaptic transmission ................................................................................................. 1
  1.1.1 Neurons and synapses ........................................................................................................ 1
  1.1.2 Synaptic transmission at chemical synapses ................................................................. 3
  1.1.3 Presynaptic active zones ............................................................................................... 3

1.2 Synaptic vesicle trafficking ....................................................................................................... 4
  1.2.1 Cellular and molecular mechanisms of synaptic vesicle trafficking ......................... 4
  1.2.2 Protein components of the synaptic vesicle trafficking apparatus ......................... 8
    1.2.2.1 Presynaptic calcium channel complexes ............................................................. 8
    1.2.2.2 Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptors (SNAREs) .................................................................................................................. 8
    1.2.2.3 NSF and soluble NSF attachment protein (SNAP) ............................................. 13
    1.2.2.4 20S fusion particle composed of SNAREs, NSF and SNAP ................................. 16
    1.2.2.5 Dynamin ............................................................................................................. 18

1.3 The genetic model system Drosophila melanogaster ............................................................. 20
  1.3.1 Advantages of using Drosophila as the model organism ............................................ 20
  1.3.2 Drosophila homologs of protein components within the synaptic vesicle trafficking apparatus ................................................................................................................................ 20
  1.3.3 Previous studies of temperature-sensitive (TS) comatose mutants ............................ 22

Chapter 2  Materials and methods ..................................................................................................... 24

2.1 Drosophila strains ....................................................................................................................... 24
  2.1.1 Cultivation of Drosophila strains ................................................................................ 24
  2.1.2 Fly stocks for generating transgenic flies ...................................................................... 24
  2.1.3 Fly stocks used in biochemistry and imaging analysis ............................................... 24

2.2 Behavior analysis ........................................................................................................................ 25

2.3 Molecular biology ..................................................................................................................... 25
  2.3.1 Extraction of Drosophila genomic DNA ..................................................................... 25
  2.3.2 Polymerase chain reaction (PCR) ............................................................................... 25
  2.3.3 Generation of DNA constructs carrying EGFP or tdTomato ..................................... 26

2.4 Drosophila genetics ................................................................................................................... 26
  2.4.1 Generation of transgenic lines carrying GFP or tdTomato tagged synaptic proteins ................................................................................................................................. 26
  2.4.2 Screening for transgenic lines ...................................................................................... 29

2.5 Biochemistry ............................................................................................................................. 31
2.5.1 Co-immunoprecipitation .......................................................... 31
2.5.2 Western blotting ................................................................. 32
2.5.3 Immunocytochemistry .......................................................... 32
2.5.4 Antibodies ........................................................................... 33
2.6 Microdissection and preparation .............................................. 34
2.6.1 Larval preparation ............................................................... 34
2.6.2 Adult dorsal longitudinal flight muscle (DLM) preparation .......... 36
2.7 Imaging .................................................................................. 37
2.7.1 Epifluorescence microscopy .................................................. 37
2.7.2 Confocal microscopy ............................................................ 37
2.7.3 Live-imaging ........................................................................ 37
2.7.3.1 Live preparation and imaging ............................................ 37
2.7.3.2 Fluorescence recovery after photobleaching (FRAP) ............... 38
2.7.3.3 Fluorescence resonance energy transfer (FRET) ...................... 38
2.8 Data analysis ......................................................................... 38
2.8.1 Quantification of activity-dependent redistribution ................. 38
2.8.2 FRAP ................................................................................ 39
2.8.3 FRET ................................................................................ 39

Chapter 3  In vivo functions and interactions of NSF and SNAP in synaptic vesicle trafficking and neurotransmitter release .................................................................................. 41

3.1 Introduction .......................................................................... 41
3.2 Results .................................................................................. 41
3.2.1 Accumulation of protein complexes containing SNAREs, dNSF1 and SNAP upon inactivation of dNSF1 ................................................................. 41
3.2.2 Presynaptic localization of endogenous dNSF1 at native synapse .......... 43
3.2.3 dNSF1 redistributes to periactive zone regions of the presynaptic plasma membrane in comatose .................................................................................. 43
3.2.4 Redistribution of dNSF1 is activity-dependent.................................. 45
3.2.5 Activity-dependent redistribution of dNSF1 results in a drastic decrease in dNSF1 mobility .................................................................................. 47
3.2.6 Activity-dependent redistribution SNAP ....................................... 49
3.2.7 Redistributed SNAP exhibits reversible association with the PAZ .......... 51
3.2.8 In vivo interaction between dNSF1 and SNAP revealed by FRET ............. 53
3.3 Discussion ............................................................................. 56

Chapter 4  In vivo Fluorescence Resonance Energy Transfer (FRET) analysis of synaptic protein interactions during neurotransmitter release ................................................................. 61

4.1 Introduction .......................................................................... 61
4.2 Objectives of the current FRET study ....................................... 65
4.3 Methodology for in vivo FRET analysis in Drosophila .................. 65
4.3.1 Selection of fluorophores to serve as the FRET pair .................... 65
4.3.2 Generation of transgenic lines for in vivo FRET analysis ............. 67
4.3.2.1 Generation of recombinant chromosomes containing a transgene insertion and a loss-of-function mutation .................................................................67
4.3.2.2 Selection of a GAL4 driver for neuronal expression of fluorescent fusion proteins ...........................................................................................................67
4.3.2.3 Crosses to generate transgenic flies co-expressing the FRET donor and acceptor in the nervous system .................................................................68
4.3.3 FRET analysis based on the 3-cube FRET measurement .................................................................70
4.3.3.1 The 3-cube FRET algorithm ..............................................................................70
4.3.3.2 Image acquisition, processing and data analysis ............................................72
4.3.3.3 Error control and correction ............................................................................72
4.3.3.4 Preliminary control experiments .....................................................................73
4.3.4 FRET analysis based on Donor Dequenching .......................................................75
4.3.4.1 Image acquisition ...........................................................................................75
4.3.4.2 Image processing and data analysis ...................................................................75
4.4 Results and conclusions ............................................................................................76
4.4.1 Detection of in vivo FRET at Drosophila larval and adult neuromuscular synapses. .....76
4.4.2 Detection of in vivo FRET using 3-Cube and Donor Dequenching FRET measurements ...........................................................................................78
4.4.2.1 Consistency between 3-Cube and donor dequenching FRET methods ....78
4.4.2.2 Detection sensitivity of 3-Cube and donor dequenching FRET measurements .................................................................80
4.4.3 In vivo interaction among SNAREs, NSF and SNAP characterized by FRET .....82
4.4.3.1 Orientations of NSF and SNAP in assembly within the 20S particle .........82
4.4.3.2 In vivo interactions between SNAP and SNAREs characterized by FRET analysis ...........................................................................................84
4.5 Discussion ................................................................................................................85
4.5.1 Significance of the current FRET study ..................................................................85
4.5.2 Interpretation of negative results ..........................................................................85
4.5.2.1 Negative FRET detection between dNSF1 and dSNAP under wild-type conditions ...........................................................................................85
4.5.2.2 Negative FRET detection between SNAREs and dSNAP .........................86
4.5.3 Biological meaning of results from the current FRET study .................................86
4.5.3.1 In vivo evidence for the accumulation of 20S fusion complex containing SNAREs, dNSF1 and dSNAP .................................................................86
4.5.3.2 In vivo evidence for the arrangement of subunits in 20S fusion particles .87
4.5.3.3 Conserved dNSF1 function at larval and adult developmental stages .......87
Chapter 5 Discussion .........................................................................................................89
5.1 Implications of the project ........................................................................................89
5.1.1 A model for spatial arrangement of protein functions with respect to presynaptic active zones ......................................................................................89
5.1.2 Live Imaging, an important research methodology in cell biology .................89
5.1.3 In vivo FRET analysis ...........................................................................................90
5.2 Future Directions

5.2.1 Identification of binding partners for dNSF1 at the PAZ

5.2.2 Improve detection sensitivity of \textit{in vivo} FRET

Bibliography
LIST OF FIGURES

Figure 1.1 Illustration of a neuron and a chemical synapse ...................................................... 2
Figure 1.2 Synaptic vesicle trafficking ..................................................................................... 5
Figure 1.3 Functional proteins involved in synaptic vesicle trafficking ................................... 7
Figure 1.4 Structure of the SNARE core complex I ............................................................... 10
Figure 1.5 Structure of the SNARE core complex II .............................................................. 12
Figure 1.6 Structure of NSF .................................................................................................... 15
Figure 1.7 Structure of the yeast homolog of α-SNAP, Sec17p ............................................. 16
Figure 1.8 20S fusion particle organization ............................................................................ 17
Figure 1.9 Domain structure and function of dynamin ........................................................... 19
Figure 1.10 Accumulation of SDS-resistant SNARE complexes in comatose^{ST17} at the restrictive temperature .......................................................... 23

Figure 2.2 Drosophila Larval neuromuscular system .............................................................. 30
Figure 2.3 Dissected Drosophila 3rd instar larval preparation ................................................ 35
Figure 2.4 Illustration of Drosophila dorsal longitudinal flight muscles (DLMs) ................. 36

Figure 3.1 Co-immunoprecipitation of endogenous core complexes containing dNSF1, SNAP and SNAREs ................................................................. 42
Figure 3.2 Confocal immunofluorescence images of endogenous dNSF1 localization at larval neuromuscular synapses ................................................................. 44
Figure 3.3 Activity-dependent redistribution of dNSF1 in comatose ..................................... 46
Figure 3.4 FRAP analysis of dNSF1 mobility ....................................................................... 48
Figure 3.5 Activity-dependent redistribution of SNAP in comatose ...................................... 50
Figure 3.6 FRAP analysis of SNAP mobility ......................................................................... 52
Figure 3.7 In vivo interaction between dNSF1 and dSNAP revealed by 3-Cube FRET measurement ......................................................................................................... 55

Figure 3.S1 Loss of dNSF1 staining in com^{CLP1} ................................................................. 57
Figure 3.S2 Lack of redistribution of dNSF1^{ST17}-EGFP under the permissive condition of
comatose mutation. .................................................................58

Figure 3.S3 Transient redistribution of EGFP-dSNAP upon 20 Hz stimulation .............58
Figure 3.S4 PAZ localization of EGFP-dSNAP after activity-dependent redistribution in
comatose. ...............................................................................................59
Figure 3.S5 PAZ localization of t-SNAREs upon 80 Hz stimulation in comatose. ..........60

Figure 4.1 Spectral properties favoring CFP and DsRed as FRET partners..................71
Figure 4.2 Preliminary control experiments. ...............................................................73
Figure 4.3 Detection of in vivo FRET using 3-Cube FRET at Drosophila third-instar larval
and adult DLM neuromuscular synapses.........................................................77
Figure 4.4 Detection of in vivo FRET using the 3-Cube FRET and donor dequenching
methods........................................................................................................79
Figure 4.5 Detection sensitivity of the 3-Cube FRET and donor dequenching measurements.
......................................................................................................................81
Figure 4.6 In vivo orientation of dNSF1 and dSNAP in assembly with each other suggested
by FRET.........................................................................................................83
Figure 4.7 In vivo interactions between SNAREs and SNAP characterized by FRET. .......84
LIST OF TABLES

Table 4.1 Properties of the best fluorescent protein variants.................................................. 67
Table 4.2 Recombinant chromosomes containing a transgenic insertion and a loss-of-function mutant. ........................................................................................................................................... 67
Table 4.3 Possible synaptic proteins interactions examined through the 3-Cube FRET measurement. ........................................................................................................................................... 74
Table 4.4 FRET pair examined with the donor-dequenching measurement........................... 76
ACKNOWLEDGEMENTS

First I’d like to thank my advisor, Dr. Richard Ordway, who is also the head of the Genetics Program at PennState. Rick has helped me all the way through the years of my Ph.D training. In fact, it was him who recruited me into the Genetics Program at PennState in the very beginning. Later, he offered me the precious opportunity to join his lab and work on this project. Not only that Rick has always been a great research advisor who guided me through difficulties in project development with lots of teaching, discussion, supporting and encouragements, he has also been a mentor and a close friend who I turned to for advice in my life.

Second, I’d like to thank Dr. Fumiko Kawasaki, a second mentor of mine in research. Fumi has taught me a great deal about how to perform experiments: from the simplest plasmid DNA purification to the most challenging technique of adult fly dissection and from doing fly work to performing confocal fluorescent imaging. Fumi has also set up a great example of a highly devoted scientist with so much enthusiasm for research, who is always organized, consistent and systematic in her work. Importantly, the work of Fumi has been instrumental in providing a foundation for the live imaging studies presented in this dissertation.

Third, I’d like to thank my committee members: Dr. Douglas R. Cavener, Dr. Wendy Hanna-Rose, Dr. Zhi-Chun Lai and Dr. Aimin Liu. I appreciate them for serving on my committee and for their invaluable suggestions and insightful comments.

Fourth, I’d like to thank Mrs. Huaru Yan, our research associate and lab manager. Yan has always taken good care of the lab and has always been helpful and supportive in my research. Yan has also done a great job as the administrative of the Genetics Program, from which I personally benefited a lot.

Next, I’d like to thank all the past and current members of the Ordway Lab, Beiyan Zou, Janani Irye, Lisa Posey, Shaona Acharjee and many others. This work and my training have benefited from the development of in vivo FRET analysis and the generation of research materials through prior unpublished work in the Ordway lab. Moreover, I thank these people for lots of good discussions along my project development and for creating such a warm, friendly and supportive atmosphere in the lab.

I’d also like to thank the Fluorescence Imaging Facility at PennState for helping me on my confocal fluorescence imaging experiments, the Nucleotide Core Facility at PennState for performing DNA sequencing and the Mass Spectrometry Facility at PennState for performing protein sample analysis.

Last but not least, I’d like to thank my husband, Peng Li, my mother, Lianshi Guo and my father, Aiwu Yu for loving me, understanding me and taking good care of me. Especially, my Mom came to the States to stay with us during my pregnancy and continued to help take care of the baby for 6 months. This is a big sacrifice for her and my Dad, because they had to
separate for almost 1 year. Finally, I’d like to thank all my friends and classmates at PennState, Xin Ye, Yi Chen, Wei Zhang, Xianlong Meng, Shihan He, Moran He, Zhao Jin, Xin Zhou and many others, for supporting me through difficult times during my Ph. D years.

Thank you all.
Chapter 1  Introduction

1.1 Chemical synaptic transmission

1.1.1 Neurons and synapses

Neuron, or nerve cell, is a highly specialized cell type that realizes functions of the nervous system. Each neuron has three morphologically defined compartments: the soma (cell body), dendrites and the axon (Figure 1.1). The cell body of a neuron is similar to other types of cells in many ways. It contains a single nucleus as well as many other typical cytoplasmic organelles and functions as the cell metabolism center. Also, the soma is the site for neural signal integration. Dendrites and the axon are cytoplasmic extensions from the soma, which also determine the polarity of a neuron. Usually, a neuron has multiple short dendrites branching out of the surface of cell body to receive signals from nearby neurons. Therefore, dendrites are called afferent processes. In contrast, a neuron has only one axon to transmit signals away from the cell body to the next cell. Axons are usually elongated projections surrounded by myelin sheaths, and they are also known as efferent fibers. At the distal end, axons form functionally specialized terminals on dendrites of other neurons or on the surface of a muscle or gland cell. These terminals are known as synapses, where signal communications within the neuronal network take place.

There are two major types of synapses, namely, chemical and electrical synapses, categorized according to the mechanism of signal transmission from the presynaptic nerve ending to the postsynaptic cell. At electrical synapses, which are rare in vertebrates, the membranes of the two adjacent cells form tight contact through specialized intercellular connections known as gap junctions, which allow a nerve impulse (or an action potential) to pass swiftly and reliably from the presynaptic nerve ending to the next cell. On the other hand, chemical synapses are more complicated structures of no physical continuity. That is, the pre- and postsynaptic cells are separated by a minute gap, known as the synaptic cleft, which prevents direct electrical transmission of the action potential (Figure 1.1). Instead, neurotransmission is achieved through releasing and reception of chemical transmitter by the pre-and postsynaptic cells.
Figure 1.1 Illustration of a neuron and a chemical synapse. A) Illustration of a neuron. Adapted from University of Texas Vertebrate Physiology (Bio365r) course website. www.utexas.edu/courses/bio354r/documents/about/about.php B) Illustration of a chemical synapse. Adapted from www.umpi.maine.edu/~stump/psybio.html
Chemical synapses can be categorized into excitatory and inhibitory types according to the effect of neurotransmission on the probability of action potential firing of the postsynaptic cell. For examples, the most prevalent transmitter, glutamate, is excitatory at over 90% of the synapses in human brain. The next prevalent transmitter, GABA, is inhibitory at more than 90% of synapses that don’t use glutamate. Other neurotransmitters, such as acetylcholine, are used at both excitatory and inhibitory synapses. Besides, neurotransmission at some synapses activates complex metabolic pathways in the postsynaptic cell to produce effects that can’t be categorized as either excitatory or inhibitory.

1.1.2 Synaptic transmission at chemical synapses

As described above, a chemical synapse is composed of pre- and postsynaptic compartments separated by a synaptic cleft. Another important structural feature is that small (~40-50 nm in diameter), membrane-bound organelles known as synaptic vesicles are packed at high densities in presynaptic compartments. These vesicles are filled with neurotransmitter molecules and fuse to specialized regions of presynaptic plasma membrane, known as the active zone, upon arrival of action potentials. Released neurotransmitters diffuse across the synaptic cleft and bind to specific receptors located on the postsynaptic membrane. Postsynaptic transmitter receptors can be categorized into ionotropic receptors (ligand-gated ion channels) and metabotropic receptors (G-protein linked receptors). When activated by neurotransmitters, both types of receptors open ion channels, through which ions flow and change the local membrane potential to produce depolarization (excitation) or hyperpolarization (inhibition) of the postsynaptic cell. Thus, a presynaptic neuronal signal is transmitted to the postsynaptic cell and propagated further down through the neuronal network.

Chemical synaptic transmission is an elaborate process carried out by symphonic functioning of multiple cellular components, including the synaptic vesicle fusion apparatus. The cellular and molecular mechanisms underlying synaptic vesicle trafficking within the presynaptic compartment has been the focus of this dissertation, and will be discussed in 1.2.

1.1.3 Presynaptic active zones

Active zones are specialized regions of presynaptic plasma membrane where neurotransmitter containing synaptic vesicles selectively fuse. Located precisely opposite to the synaptic cleft, active zones are platforms for rapid synaptic vesicle fusion upon calcium triggering. At active zones, the plasma membrane is decorated by electron-dense, biochemically insoluble cytomatrix (cytoskeleton matrix at the active zone; CAZ) which typically measures a few hundred nanometers in diameter and displays variable morphologies at different types of synapses. Synaptic vesicles cluster around the electron-dense projections.

Low solubility of active zone materials has thwarted functional characterizations of active zone proteins. However, investigations involving different model organisms have identified 6 large proteins [reviewed in (Dresbach et al., 2001)], each encoded by multiple genes, to be fundamental components of the CAZ, which are essential for organizing the assembly of cytomatrix components Piccolo and Bassoon, coiled-coil proteins ERCs
(ELKS/Rab3-interacting molecule/CAST), SH3-domain protein RIM-Bps and α-Liprins. Among which, RIMs are central elements of active zones because they bind directly to Munc13, ERCs (Ohtsuka et al., 2002), RIM-BPs (Wang et al., 2000) and α-Liprins (Schoch et al., 2002). In addition, all ERCs bind to α-liprins (Ko et al., 2003), Piccolo and Bassoon (Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004). Binding of these proteins probably form a large single complex at the active zone, which links to synaptic vesicles through interaction with vesicle proteins Rab3 (Wang et al., 1997; Wang et al., 2000) and synaptotagmin 1 (Coppola et al., 2001; Schoch et al., 2002).

1.2 Synaptic vesicle trafficking

1.2.1 Cellular and molecular mechanisms of synaptic vesicle trafficking

Synaptic vesicles undergo a trafficking cycle within nerve terminals. After years of study, it is well-accepted that synaptic vesicle trafficking can be divided into several sequential steps including docking, priming, fusion and fission (Figure 1.2). Synaptic vesicles loaded with neurotransmitters cluster in a reserve vesicle pool away from active zones. Some vesicles can be mobilized out of this pool, and the process of them coming into close proximity to the presynaptic plasma membrane is defined as docking. After making contact with the target membrane, docked vesicles are primed through a series of chemical reactions and become competent for immediate neurotransmitter release upon the Ca$^{2+}$ signal triggering. When a nerve impulse (an action potential) arrives at the nerve terminal, the change in membrane potential opens voltage-gated calcium channels. Influx of extracellular Ca$^{2+}$ increases in the local Ca$^{2+}$ concentration and triggers fusion-pore opening and membrane fusion of primed vesicles. After release of neurotransmitters, plasma membranes internalize to form new synaptic vesicles through clathrin-mediated endocytosis. Sometimes, neurotransmitters are released through another mechanism known as the “kiss-and-run” pathway, where a fusion pore is opened transiently and reseals after releasing neurotransmitters into the synaptic cleft. After endocytosis, vesicles are reacidified, refilled with neurotransmitters and recycled either locally to the readily releasable pool or through the reserved pool for subsequent rounds of fusion (Koenig and Ikeda, 1996; Pyle et al., 2000; Richards et al., 2000).
**Figure 1.2 Synaptic vesicle trafficking.** At the presynaptic terminal, vesicles mobilize out of a reserve pool and dock onto the plasma membrane. Docked vesicles are primed for fusion and await Ca\(^{2+}\) triggering in a readily-releasable pool. In response to Ca\(^{2+}\) influx upon the arrival of action potentials, primed vesicles immediately fuse to release neurotransmitters. After fusion, new vesicles fission from the plasma membrane and recycled either locally in the releasable pool or through the reserve pool for subsequent rounds of fusion.
Several proteins function at the core of synaptic vesicle trafficking apparatus. Besides the presynaptic calcium channel complex that allows Ca\(^{2+}\) influx in response to action potentials, vesicle priming and subsequent membrane fusion involve functions of soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptors (SNAREs). Specifically, a vesicle-associated SNARE protein, v-SNARE, interacts with two target membrane SNARE proteins, t-SNAREs, to form a loose complex during priming, which upon Ca\(^{2+}\) triggering undergoes conformational changes and becomes tightly associated. This conformational change of the SNARE complex and energy released during the process are believed to catalyze membrane fusion. The term “trans-SNARE complex” refers to the loose conformation when the v- and t-SNAREs sit on separate membranes. In contrast, the term “cis-SNARE complex” refers to the fully assembled conformation after complete vesicle fusion when the v- and t-SNAREs are located within the same membrane (Figure 1.3). Afterwards, cis-SNARE complexes are disassembled to produce free SNAREs for future rounds of fusion. This process is essential for maintaining the synaptic vesicle trafficking cycle and requires functions of two soluble protein factors, N-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment protein (SNAP). During clathrin-mediated endocytosis, dynamin functions at early stages to regulate clathrin pit maturation, and at later stages to directly catalyze membrane fission. Functions and interactions of protein components during the synaptic vesicle trafficking cycle are discussed in details in 1.2.2.
Figure 1.3 Functional proteins involved in synaptic vesicle trafficking. Docked vesicles are primed for fusion through a series of chemical reactions, during which the v-SNARE forms trans-SNARE complexes with t-SNAREs. Influx of Ca\textsuperscript{2+} through the Voltage Gated Calcium Channel (VGCC) triggers fusion of primed vesicles and the resultant cis-SNARE complexes are subsequently disassembled through functioning of NSF and SNAP. Thus, free SNAREs are recycled for future rounds of fusion and new vesicles fission from the plasma membrane through dynamin-dependent endocytosis.
1.2.2 Protein components of the synaptic vesicle trafficking apparatus

1.2.2.1 Presynaptic calcium channel complexes

Presynaptic calcium channels are important synaptic components indispensable for neurotransmission. Upon arrival of action potentials, change of membrane potential opens voltage gated calcium channels (VGCC) and allows calcium influx into the presynaptic compartment to trigger evoked transmitter release. Intensive research effort has revealed the structures, functions and electrophysiological properties of these channels. Six types of VGCCs, L-type, N-type, P/Q type, R-type and T-type, express in neurons. Among them, N-type and P/Q type are the primary presynaptic calcium channels involved in vesicle fusion and neurotransmitter release. Structurally, these channels consist of several subunits including \( \alpha_1 \), \( \beta \) and \( \alpha_2 \delta \) subunits (Arikkath and Campbell, 2003). \( \alpha_1 \) subunit is the pore forming and primary structural subunit of the channel. Accessory subunits \( \beta \) and \( \alpha_2 \delta \) have been found to regulate trafficking and targeting of calcium channels and are also involved in modulation of channel functions (Arikkath and Campbell, 2003).

*Drosophila* \( \alpha_1 \) subunit is encoded by gene *cacophony* (*cac*), which has been shown to localize specifically to the presynaptic active zone (Kawasaki *et al*., 2004). Transgenic expression of fluorescently tagged \( \alpha_1 \) subunit has allow live imaging of functional presynaptic calcium channels *in vivo* and also has been efficiently used as an active zone marker in both live imaging and immunocytochemistry studies at native synapses.

1.2.2.2 Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptors (SNAREs)

First introduced in 1993, the term “SNARE”, stands for Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor. SNAREs are a large superfamily of small membrane proteins consisting of more than 60 members in yeast and mammalian cells (Karp, 2002; Jahn and Scheller, 2006). SNARE proteins are thought to be essential for membrane fusions during all the trafficking steps of eukaryotic secretory pathways. Identification of neuronal SNAREs that mediate synaptic vesicle fusion during neurotransmission (Söllner *et al*., 1993a; Söllner *et al*., 1993b) suggests a universal SNARE-mediated mechanism of vesicle fusion common to both constitutive and regulated membrane trafficking.

Structurally, all SNARE proteins share an evolutionarily conserved domain of about 60 amino acids arranged in heptad repeats, of which the secondary structure is a coiled-coil of \( \alpha \)-helix. This structural feature known as the SNARE motif is functionally important because it mediates the association of SNAREs into core complexes (discussed later) (Terrian and White, 1997; Weimbs *et al*., 1997; Weimbs *et al*., 1998). In addition, most SNAREs have a C-terminal transmembrane (TM) domain that is linked to the SNARE motif by a short linker. Positioned N-terminal to the SNARE motif, many SNAREs have independently folded domains that mediate additional protein-protein interactions. A subset of SNAREs lacks the transmembrane domain, and most of these achieve membrane anchorage through hydrophobic post-translational modifications such as palmitoylation.
Different sets of SNAREs are found on two opposing membranes that are destined to fuse. Based on the membrane type they associate with, SNAREs are classified as vesicle associated, v-SNAREs, and target membrane associated, t-SNAREs (Söllner et al., 1993b; Hay et al., 1998). However, this terminology is not applicable to homotypic fusion events. Also, all SNAREs are evolutionarily related to each other and localization of trafficking vesicle or target membrane does not always correlate with structurally identified SNARE subfamilies (Weimbs et al., 1998; Fasshauer et al., 1998b). Because crystal structures show that a central residue in the SNARE motif is either a glutamine or an arginine, a preferred classification categorizes SNAREs as Q- and R-SNAREs based on the type of this central amino acid (Fasshauer et al., 1998b).

To date, the best characterized SNARE proteins are the ones involved in synaptic exocytosis. Neuronal SNAREs include synaptobrevin (SYB) which is also known as vesicle-associated membrane protein or VAMP, syntaxin (SYX), and synaptosomal-associated protein of 25 kDa (SNAP-25). SYB or VAMP was initially discovered as an integral membrane protein of 18kDa present in small synaptic vesicles in neurons (Trimble et al., 1988; Baumert et al., 1989). In human, 2 highly conserved homologous SYB 1 and 2, are encoded by separate genes SYB1 and SYB2, respectively (Archer et al., 1990). SYX (35kDa) and SNAP-25 (25kDa) were initially identified as neuronal proteins that are concentrated on the plasma membrane at synaptic sites. Both proteins were implicated in synaptic vesicle priming at presynaptic active zones (Oyler et al., 1989; Bennett et al., 1992). SYB was classified as a v-SNARE due to its vesicular anchoring, while SYX and SNAP-25 were categorized as t-SNAREs because of their plasma membrane localizations (Söllner et al., 1993b).

SYB has a single SNARE motif, which makes up most of the cytosolic portion of this protein and links to its C-terminal membrane anchor. SNAP-25 contains two different SNARE motifs located at its N- and C-termini. These two coiled-coil domains are connected by a flexible linker that contains 4 palmitoylated cysteine residues to mediate membrane anchorage. SYX has a single SNARE motif, the cytosolic H3 domain, flanked by a C-terminal transmembrane domain and an N-terminal cytosolic fragment containing three coiled-coil domains, Ha, Hb and Hc (Fernandez et al., 1998). These α-helices are structurally similar to SNARE motifs and can interact with the H3 domain to fold SYX into a closed conformation unable to bind other SNAREs (Calakos et al., 1994; Pevsner et al., 1994). Switching between the open and closed conformations of SYX requires the function of additional regulatory factors, including nSec-1, Rab and Rab effectors (Dulubova et al., 1999; MacDonald et al., 2010).

When associated in a 1:1:1 stoichiometry, SYB, SYX and SNAP-25 form a SNARE complex also known as the core complex. Assembly of the ternary core complex is through interactions of SNARE motifs of the three SNAREs. Specifically, the core complex is a four helical bundle with SYB contributing its cytosolic coiled-coil domain, SYX contributing its H3 domain and SNAP-25 contributing both C- and N-terminal coiled-coils. The coiled-coil domains of SNAREs associate in a parallel alignment. That is, the N-termini are adjacent to one another with the C-termini adjacent to one another on the opposite end (Hanson et al., 1997; Lin and Scheller, 1997; Poirier et al., 1998a). Such alignment brings the
umtransmembrane domains of SYB and SYX close together and thus puts the synaptic vesicle membrane into close opposition to the plasma membrane (Figure 1.4).

**Figure 1.4 Structure of the SNARE core complex I.** Assembled of SYB, SYX and SNAP-25 in a 1:1:1 stoichiometry, the SNARE core complex is a four α-helical bundle with SYB contributing its cytosolic coiled-coil domain, SYX contributing its H3 domain and SNAP-25 contributing both C- and N-terminal coiled-coils. The coiled-coil domains of SNAREs are aligned in parallel from the N- to C-termini, and such alignment put the synaptic vesicle membrane into close opposition of the plasma membrane. Modified from Rizo and Südhof (2002).
Full assembly of the core complex may go through several conformational intermediates. Binding of SYX and SNAP-25 to form a binary receptor complex on the plasma membrane awaiting the addition of SYB may be the first step of core complex nucleation. (Fasshauer and Margittai, 2004; Wiederhold et al., 2010). During vesicle priming, a loose ternary SNARE complex is formed in trans between the membranes. Frequently referred to as the trans-SNARE complex, this conformation is formed through partial association of the SNARE motifs at the N-terminal ends, while the membrane proximal C-termini of SNARE motifs and the transmembrane domains of SYX and SYB remain disassociated. Complete core complex nucleation is through a rapid N- to C-terminal zippering of SNARE motifs triggered by a fusion signal (Sørensen et al., 2006).

Fully assembled core complexes, corresponding to cis-SNARE complexes formed after complete synaptic vesicle fusion, are extremely energetically favorable and exhibit extraordinary stability. Specifically, the core complex resists denaturation by SDS (Hayashi et al., 1994) and exhibits great heat tolerance with a melting temperature of greater than 90 °C (Fasshauer et al., 1998a; Poirier et al., 1998a; Yang et al., 1999). In monomeric or partially assembled states, the 3 neuronal SNAREs are substrates of clostridial neurotoxins (Pellizzari et al., 1999). Cleavage of SYB by tetanus toxin and botulinum toxin types B, D, F and G, SNAP-25 by botulinum toxins A, C and E or SYX by botulinum toxin C produces rapid inhibition of neurotransmission (Pellizzari et al., 1999). However, full assembly of core complexes results in resistance to prototypical cleavage by these toxins, because cleavage sites are buried within the intertwined bundle of helices (Hayashi et al., 1994).

The X-ray crystal structure of the fully assembled SNARE core complex showed that the coiled-coil bundle can be divided into 16 stacked layers of interacting side chains, denoted from -7 to +8 (Figure 1.5). The interaction is mostly via hydrophobic forces except for layer 0 at the center, where SYX and SNAP-25 arginine residues interact with the SYB glutamine residue via ionic bonding (Sutton et al., 1998) (Figure 1.5). Layer 0 also possesses the greatest diameter of the layers and is speculated to be essential for proper alignment of the helices during zippering or in disassociation of the complex (Ossig et al., 2000; Scales et al., 2001; Sørensen et al., 2006).
Figure 1.5 Structure of the SNARE core complex II. A) 16 stacked layers of interacting side chains, from -7 to +8, mediate the interactions between the SNARE coiled-coil domains. B) At layer 0, SYX and SNAP-25 contribute glutamine residues and SYB contributes an arginine to form ionic bonding. Modified from Sutton et al. (1998).
The above biochemical, structural and functional data support the following model of SNARE-mediated synaptic vesicle membrane fusion. Membrane fusion is initiated via assembly of the SNARE core complex. The SYX H3 domain is liberated from association with the Habc domain and binds to SNAP-25 to form a binary receptor complex to which SYB is added. During priming, the ternary SNARE complex is partially assembled via association of the N-termini of SNARE motifs, and undergoes rapid zippering towards the C-termini upon Ca2+ triggering. Helical extension proceeds beyond the core complex into the linker regions between SNARE motifs and transmembrane domains (Stein et al., 2009), which brings the vesicle and plasma membrane into contact and disrupts the lipid bilayer structure to initiate membrane merger. The repulsion force between the phospholipids is likely overcome by the free energy released during formation of this extremely stable coiled bundle. After membrane fusion, resultant cis-SNARE complexes are disassembled to release free SNAREs to participate in future rounds of vesicle priming and fusion. Disassembly of this stable protein complex requires the ATPase NSF (N-ethylmaleimide sensitive factor) and its cofactor SNAP (soluble NSF attachment protein).

### 1.2.2.3 NSF and soluble NSF attachment protein (SNAP)

N-ethylmaleimide (NEM) sensitive factor (NSF) was first discovered as a soluble protein factor responsible for NEM sensitivity of in vitro vesicular transport between successive cisternae of the Golgi apparatus (Glick and Rothman, 1987; Block et al., 1988; Malhotra et al., 1988; Orci et al., 1989). Poisoning the in vitro transport system with NEM blocks vesicle fusion (Wilson et al., 1989). Subsequent studies reveal that NSF is also required for vesicular transport between endoplasmic reticulum (ER) and Golgi apparatus and between trans-Golgi networks at the vesicle fusion stage (Beckers et al., 1989; Diaz et al., 1989). Later, NSF was also implicated in synaptic vesicle exocytosis (Söllner et al., 1993a; Söllner et al., 1993b).

Soluble NSF attachment proteins (SNAPs) are a family of small soluble proteins required for intracellular membrane fusion in all eukaryotic organisms. Three related isoforms, termed α- (35kDa), β- (36kDa) and γ-SNAP (39kDa), were initially purified from bovine brain cytosol according to their transport factor activities (Clary et al., 1990). Examined with the in vitro Golgi transport reaction (Balch et al., 1984), transport activity of SNAP was required at the same step as NSF. Three SNAPs were able to bind NSF to Golgi membranes (Clary et al., 1990). Later, SNAREs were identified as plasma membrane receptors and substrates for NSF-SNAP (Söllner et al., 1993a). More specifically, SNAP acts as a cofactor to bridge the binding of NSF to SNAREs (Weidman et al., 1989). Subsequent disassociation of plasma membrane SNARE complexes is via NSF-dependent ATP hydrolysis, releasing free SYB, SYX and SNAP-25 to participate in future rounds of vesicle priming and fusion.

Prior to purification of the mammalian NSF and SNAP, the yeast homologs Sec18p and Sec17p were identified as essential factors in various steps of the secretory pathway (Novick et al., 1980; Novick et al., 1981; Eakle et al., 1988). Moreover, NSF and α-SNAP were found to be functionally equivalent to Sec18p and Sec17p respectively (Wilson et al., 1989; Clary et al., 1990), suggesting that NSF and SNAPs are components of a general intracellular membrane fusion apparatus common to all eukaryotic cells.
NSF belongs to the AAA⁺ (ATPase associated with various cellular activities) protein superfamily. Some of these ATPase were shown to act as molecular chaperones to modulate the folding and oligomerization of protein complexes. Structurally, NSF is a homohexamer of 76kDa subunits. Each subunit is composed of an N-terminal domain and 2 tandem ATPase domains, D1 and D2 (Tagaya et al., 1993; Whiteheart et al., 1994; Fleming et al., 1998) (Figure 1.6). Functional and structural studies suggest an essential function of the N-terminal domain is to bind SNAP and SNARE complexes. The C-terminal D2 domain has been shown to mediate hexamerization of the NSF subunits while the center D1 ATPase domain is responsible for SNARE complex disassembly (Whiteheart et al., 1994; Nagiec et al., 1995). Hexameric NSF is a hollow 10 ×16 nm cylinder with the subunits aligned in a way that the D1 and D2 rings stacked on top of each other surrounded by N-terminal domains (Figure 1.6). The conformation of this hexamer changes with the ATP-ADP cycle of NSF. Importantly, the N-terminal domains hold out at the feet of D1 domain when NSF is bound with ATP (Figure 1.6). This conformation facilitates binding of the N-terminus to SNAP and SNAREs. In contrast, upon ATP hydrolysis (bound with ADP), the N-terminal domains draw in and hold tight to both D1 and D2 rings (Figure 1.6) and disassociate from SNAREs (Hanson et al., 1997).
Figure 1.6 Structure of NSF. A) Monomeric NSF contains an N-terminal domain and 2 tandem ATPase domains, D1 and D2. B) Hexameric NSF is a hollow 10×16 nm cylinder with the D1 and D2 rings stacked on top of each other surrounded by N-terminal domains. In bound with ATP, the N-terminal domains of NSF hold out at the feet of the D1 ring, whereas in bound with ATP, the N-terminal domains draw in and hold tight to both D1 and D2 rings. Modified from Hanson et al. (1997).
Revealed by X-ray crystallography, the yeast homolog of α-SNAP, Sec17p, is a 14-helix α/α protein composed of 2 principle domains. The N-terminal 9 α-helices are arranged in antiparallel to form a sheet-like structure and the 5 α-helices at the C-terminus form a globular bundle (Figure 1.7) (Rice and Brunger, 1999). Site mutation and truncation of α-SNAP amino acid sequence have led to insights regarding how α-SNAP interacts with SNARE complexes and NSF. According to Marz et al. (Marz et al., 2003), the SNARE complex binding surface of α-SNAP is the concave face of the N-terminal twisted sheet domain, of which the curvature complements that of the convex surface of the SNARE complex. Although α-SNAP possesses distinct N- and C- terminal NSF binding sites, the C terminus has been shown to play a critical role in stimulating NSF ATPase activity (Barnard et al., 1997; Wimmer et al., 2001).

Figure 1.7 Structure of the yeast homolog of α-SNAP, Sec17p. Illustrated by the ribbon drawing, Sec17p is a 14-helix α/α protein composed of 2 principle domains. The N-terminal 9 α-helices are arranged in antiparallel to form a twisted sheet-like structure, whose concave face is thought to be the SNARE complex binding surface, and the 5 α-helices at the C-terminal form a more globular bundle, which is asymmetrically disposed with respect to the N-terminal sheet. Modified from Rice et al. (1999).

1.2.2.4 20S fusion particle composed of SNAREs, NSF and SNAP

Binding of NSF and SNAP to SNAREs gives rise to a 20S fusion particle, named after its sedimentation coefficient in Svedberg units (Wilson et al., 1992). Multiple studies employing electron microscopy have revealed the sequential assembly, structure, stoichiometry and spatial organization of subunits of the 20S fusion particle (Figure 1.8). Briefly, fully assembled ternary SNARE complex possesses a rod-like structure with a tail hanging at one end, corresponding to the N-terminus of SYX. The membrane anchors of SYX and SYB are arranged at the opposing end of the rod (Hanson et al., 1997; Hohl et al., 1998b). Three α-SNAP molecules bind laterally to the SNARE rod, increasing the diameter but not the length of the rod (Hanson et al., 1997; Hohl et al., 1998b; Wimmer et al., 2001). α-SNAP binds SNAREs along the entire length of the rod in a sheath-like manner (Hohl et al., 1998b). Thus, a significant portion of SNAP is thought to participate in its interaction with the SNARE complex and this spatial arrangement may be important for translation of free energy released from NSF-dependent ATPase hydrolysis into conformational change of the SNARE rod. Moreover, α-SNAP binds SNAREs in an antiparallel orientation, thus positioning its N
terminus near the membrane and its C terminus away from the membrane to interact with NSF (Hanson et al., 1997; Hohl et al., 1998b). The SNAP-SNARE complex recruits a NSF hexamer at the distal end with respect to the SNARE membrane anchors. The double rings (D1 and D2 domains) of NSF hexamer are mostly stacked on top of the SNAP-SNARE complex with the SNARE rod partially inserted into the inner cavity of NSF, making contact with the N and D1 domains (Hohl et al., 1998b).

**20S Fusion Particle Organization**

![Diagram of 20S fusion particle organization](image)

**Figure 1.8 20S fusion particle organization.** SNAREs assemble into the rod-like core complex with a C-terminal membrane anchor made of transmembrane domains of SYX and SYB. Three α-SNAPs bind laterally to the SNARE rod in an anti-parallel fashion, positioning their N-termini close to the membrane. A NSF hexamer binds to the SNARE/SNAP complex on the membrane distal end with the N and D1 domains positioned closer to SNARE/SNAP than the D1 domain. Modified from Wimmer et al. (2001).
It is not clear exactly how NSF and SNAP operate together to disassemble the SNARE complex in the 20S fusion particle. Upon ATP hydrolysis, free energy produced by NSF D1 ATPase domain at $6 \times 32 \text{ kJ/mol}$ should be sufficient to support complete disassembly of 20S particles, including unwinding of SNARE helical bundles and disassociation of NSF and SNAP. Released free energy may be transferred through SNAP to the SNARE rod to facilitate conformational transition of one or more of the SNAREs. Relocation of the NSF N-terminal domains and possible conformational changes of SNAP during this process may also promote the disassembly.

1.2.2.5 Dynamin

Dynamin is a large oligomeric GTPase that plays an essential role in clathrin-mediated endocytosis (CME). Drosophila melanogaster contains multiple tissue-specific forms of dynamin, all encoded by the shibire gene (Chen et al., 1991; van der Biek and Meyerowrtz, 1991), which are implicated in vesicle trafficking and endocytosis in and outside of the nervous system (Takei et al., 1995; Kawasaki et al., 2000b; Praefcke and McMahon, 2004). Each subunit of dynamin is a 96kDa peptide consisting five conserved domains, including an N-terminal GTPase domain, a middle domain involved in tetramerization and higher-order self-assembly (Ramachandran et al., 2007) and intracellular targeting (Liu et al., 2008), a pleckstrin-homology (PH) domain that binds to phosphatidylinositol lipids (Yarar et al., 2008), a GTP effector domain (GED) that mediates self-assembly and assembly-enhanced GTPase activity (Sever et al., 1999) and a C-terminal proline/arginine rich domain (PRD) that binds to SH3 domain (Src homology 3 domain)-containing proteins (Schmid et al., 1998) (Figure 1.9).

According to a model proposed for dynamin function at the final stage of CME (Fig 1.9), GTP binding triggers dynamin self-assembly into rings and helical stacks of rings around the neck of clathrin-coated pits. Assembly-stimulated GTP hydrolysis triggers a concerted conformational change to pinch off nascent vesicles (Hinshaw and Schmid, 1995; Warnock and Schmid, 1996). However, recent evidence suggests another role for dynamin in CME at early stages to regulate clathrin-coated pit maturation [reviewed in (Mettlen et al., 2009)].
Figure 1.9 Domain structure and function of dynamin. A) Dynamin is composed of five conserved domains of indicated functions, including an N-terminal GTPase domain, a middle domain, pleckstrin-homology (PH) domain, GTP effector domain (GED) and C-terminal proline rich domain (PRD). B) A model proposed for dynamin to function as a mechanochemical molecule at the final stage of CME. GTP binding triggers dynamin self-assembly into collars around the neck of clathrin-coated pits, and assembly-enhanced GTPase activity triggers a concerted conformational change to provide the forces needed for membrane fission. Adapted from Mettlen et al. (2009)
1.3 The genetic model system *Drosophila melanogaster*

1.3.1 Advantages of using *Drosophila* as the model organism

*Drosophila melanogaster* is a sophisticated genetic model organism that has been widely used in biological research. *Drosophila* is an easy lab-culture organism with a short life cycle, massive reproduction, and its genetics has been intensively-studied. Its relatively small and simple genome (a hundred million base pairs) is composed of 4 pairs of chromosomes, including 1 sex and 3 autosomal chromosomes, within which 50% of proteins have mammalian homologs. Years of study has developed many tools for genome and gene manipulation of *Drosophila*, including matured methodologies for forward and reverse genetics, transgenic lines, and temporal and spatial control of expression using the UAS-GAL4 system.

Forward genetic screens in *Drosophila* have revealed numerous genes with critical roles in neurotransmission. First described by Suzuki and colleagues (Suzuki et al., 1971), one particularly fruitful approach has involved temperature-sensitive (TS) paralytic mutants, which has identified proteins required acutely in neuronal signaling. TS paralytic mutations are interesting models for analyzing *in vivo* protein functions because disruption of neurotransmission can be induced acutely at the restrictive temperature without jeopardizing other processes during development of mutant animals.

The UAS-GAL4 system adapted from yeast (Fischer et al., 1988), is a powerful approach for studying gene expression and function in *Drosophila*. Thousands of tissue specific, heat shock promoter linked or GAL80<sup>ts</sup> (McGuire et al., 2003) regulated *Drosophila* GAL4 lines have been establish to drive expression of target genes downstream of UAS sequence in a spatiotemporal specific manner.

The *Drosophila* neuromuscular synapse (also known as neuromuscular junction or NMJ) is an ideal model system to study synaptic function. Many of the proteins implicated in synaptic transmission are strongly conserved between *Drosophila* and vertebrates (typically 70-80% relative to the mammalian homologues) and are expressed at the neuromuscular synapse (Keshishian et al., 1996), suggesting fundamentally conserved mechanisms. Importantly, the system can be manipulated with genetic methods, including gene reconstruction, mutagenesis and transgenes that alter the expression pattern and function of target proteins, and is easily accessible to imaging, biochemical and electrophysiological analysis after delicate microdissection (see 2.6).

1.3.2 *Drosophila* homologs of protein components within the synaptic vesicle trafficking apparatus

*Drosophila* has two homologs of SYB. Encoded by related genes *n-syb* and *syb* respectively, neuronal-SYB (NSYB) is a neuronal homolog that is widely (perhaps ubiquitously) expressed in the *Drosophila* nervous system, whereas SYB is generally expressed in multiple tissues including abundant expression in the gut and very low expression in neurons (Chin et al., 1993; DiAntonio et al., 1993). Several lines of evidence (Sweeney et al., 1995; Deitcher
et al., 1998) point to the fact that the two homologs of Drosophila synaptobrevin may mediate target-specific membrane fusion events. That is, n-syb function is specifically required for synaptic vesicle fusion, whereas syb plays a role in constitutive membrane fusion in the organism, including general membrane addition and axonal outgrowth in the nervous system. Similarly, the Drosophila homolog SNAP-25 has a strictly neuronal expression pattern at neuronal axons and synapses, and is essential for synaptic vesicle fusion (Risinger et al., 1993; Risinger et al., 1997). On the other hand, non-synaptic fusion in neurons and constitutive and regulated fusions in other cells are mediated by a second member of the Drosophila SNAP-25 family, SNAP-24, which is encoded by a closely related gene and is expressed throughout development (Niemeyer and Schwarz, 2000). Unlike the target-specific division of function of SYB and SNAP-25 homologs, a Drosophila homolog of SYX, encoded by the gene syntaxin 1A, is ubiquitously expressed and appears to be required universally for nonneuronal secretion and neurotransmission (Schulze et al., 1995; Burgess et al., 1997).

Drosophila homologs of NSF (dNSF1) and SNAP were identified in 1994 (Ordway et al., 1994), and a second homolog of Drosophila NSF, dNSF2 was discovered in 1995 (Boulianne and Trimble, 1995; Pallanck et al., 1995b). dNSF1 and dNSF2 are encoded by distinct Drosophila genes, and show 84% amino acid identity to one another (Pallanck et al., 1995b). The dNSF1 gene is also known as comatose, which is named after the TS paralytic phenotype of mutant alleles recovered in a classic genetic screen (Siddiqi and Benzer, 1976; Pallanck et al., 1995a). The two homologs of dNSF display overlapping but different temporal expression pattern. Specifically, dNSF1 function is required for viability beginning at the adult stage of development, supported by the fact that dNSF1 is expressed at high levels in adults and at relatively low levels earlier during development, and that loss-of-function mutations in comatose lead to pharate adult lethality (Golby et al., 2001). In contrast, dNSF2 is expressed at similar levels throughout development (Pallanck et al., 1995b), and its activity is required for viability beginning at the first-instar larval stage of development (Golby et al., 2001). Moreover, dNSFs exhibit tissue-specific division of functions, that is, the essential function of dNSF1 resides primarily (perhaps solely) in the nervous system and dNSF2 is required primarily in mesoderm (Golby et al., 2001). Analysis of the TS mutation of the comatose gene (Pallanck et al., 1995a) has provided direct functional evidence of a role for dNSF1 during synaptic transmission (Kawasaki et al., 1998; Littleton et al., 1998; Tolar and Pallanck, 1998). Also, dNSF2 may play a second role in the nervous system of the organism in determining synaptic growth or development (Stewart et al., 2002; Stewart et al., 2005; Nunes et al., 2006). Despite their differing temporal and spatial requirements, the dNSF1 and dNSF2 proteins are capable of substituting for one another in ectopic expression studies (Golby et al., 2001), suggesting overlapping functional properties of the protein homologs.

Encoded by dSNAP, the single homolog of Drosophila SNAP is closely related to bovine α- and β-SNAP, and is more distantly related to bovine γ-SNAP or yeast Sec17p (Ordway et al., 1994). At the embryonic stage, expression of dSNAP is strictly within the central nervous system (Ordway et al., 1994). However, null mutations of dSNAP lead to embryonic lethality (Babcock et al., 2004), suggesting an ubiquitous requirement of SNAP activity outside the


1.3.3 Previous studies of temperature-sensitive (TS) comatose mutants

Temperature-sensitive (TS) alleles of *comatose* were initially identified in a classical genetic screen for mutations leading to paralysis at elevated temperatures (Siddiqi and Benzer, 1976). Later, the *comatose* locus was mapped to the dNSF1 gene (Pallanck et al., 1995a), which provides the first functional evidence of a role for dNSF1 during synaptic transmission. Further electrophysiological and ultrastructural analysis of *comatose* mutants demonstrated a priming role of dNSF1 in preparing docked synaptic vesicles for immediate action potential-evoked fusion (Kawasaki et al., 1998). Moreover, this priming role of dNSF1 was shown to involve dNSF1-dependent disassembly of the neural SNARE complex at the plasma membrane after vesicle fusion (Littleton et al., 1998; Tolar and Pallanck, 1998), as revealed by accumulation of SDS-resistant SNARE complex in *comatose* mutants at the restrictive temperature (Figure 1.10). A more recent study has further suggested the presynaptic active zone as the location for cis-SNARE complex disassembly and a role for dNSF1 in maintaining active zone free t-SNAREs available for vesicle priming reactions (Kawasaki and Ordway, 2009). The particular TS *comatose* allele analyzed in the current dissertation project is *comt*\textsuperscript{ST17}, of which the mutation is caused by a G274Q substitution in the D1 ATPase domain of NSF (Pallanck et al., 1995a), a region essential for SNARE complex disassembly (Nagiec et al., 1995).
Figure 1.10 Accumulation of SDS-resistant SNARE complexes in *comatose*<sup>ST17</sup> at the restrictive temperature. *Comatose* flies were kept at the permissive temperature of 20 °C or exposed to the restrictive temperature of 38 °C before decapitation. Equal amounts of head homogenates from both samples were loaded on a SDS-denaturing gel without boiling. Anti-SYX antibody was used to blot SDS-resistant SNARE complexes as well as SYX monomers. Note the elevated amount of SNARE complex and reduced amount of SYX monomer at high temperature. Protein size standards of 35, 50 and 90kDa were marked for reference.
Chapter 2  Materials and methods

2.1 Drosophila strains

2.1.1 Cultivation of Drosophila strains

Stocks of all Drosophila strains were cultured with the standard cornmeal-molasses-yeast medium. Recipe: 1000 ml Molasses, 108 g Agar, 1000 ml Cornmeal, 400 ml Brewers Yeast, 40 ml Propionic Acid, 90 ml 20% Tegosept (p-methoxy benzoic acid) in 95% ethanol and 9 L H2O.

Fly stocks and crosses producing experimental animals (used directly in behavior, biochemical or imaging analysis) were grown at 20 °C with 60% humidity in a controlled environmental room. Crosses in progress of establishing new stocks, including transgenic lines, were grown at the room temperature (around 22 °C). For the purpose of transformation rescue, sometimes crosses of transgenic expression driven by GAL4 were grown at 25 °C to reach a higher expression level.

2.1.2 Fly stocks for generating transgenic flies

Several stocks were used to establish transgenic fly lines. The egg-laying stock, of which freshly laid eggs were injected with DNA solution, is

\[ w^{176} \]

Stocks used to map the location of transgenic insertions are:

\[ w^1 \]
\[ w^1, SM5; TM3 /ap^{Xa} \]
\[ w^1, Df (1) sd / FM7C \]
\[ w^1, Sco/Cyo \]
\[ w^1; TM3/TM6B \]

All stocks are from Dr. Richard Ordway’s Lab collection (PennState University, State College, PA). A detailed crossing scheme describing functions of these stocks are provided in 2.4.1

2.1.3 Fly stocks used in biochemistry and imaging analysis

Canton-S (CS), \[ com^{	ext{ST17}} \], and all transgenic lines carrying a fluorescent fusion protein gene downstream of UAS are from our (Ordway lab) stock collection. CS was used as the wild-type strain. Elav-Gal4 was obtained from the Bloomington Stock Center, and Appl-Gal4 (Torroja et al., 1999) was kindly provided by Dr. Laura Torroja (Universidad Autonoma de Madrid, Madrid, Spain). \[ Df(3R)\Delta 229 \] (Schulze et al., 1995) was obtained from Dr. Hugo Bellen’s lab (Baylor College of Medicine, Houston, Texas), \[ SNAP-25^{124} \] (Vilinsky et al., 2002) was obtained from David Deitcher’s lab (Cornell University, Ithaca, New York) and \[ nsysb^{AP33B} \] (Deitcher et al., 1998) were obtained from Dr. Thomas Schwarz’s lab (Stanford University Medical Center, Stanford, California).
2.2 Behavior analysis

Behavior analysis were carried out as described previously (Dellinger et al., 2000; Kawasaki et al., 2000a; Kawasaki et al., 2002; Brooks et al., 2003). Two-day-old flies raised at 20 °C were tested for temperature-sensitive behavior phenotypes in groups of six. Temperature controlling was achieved through a water bath with a transparent container. A transparent plastic vials with a cotton seal was first pre-heated in the water bath to the testing temperature of 38 ºC. Then a group of flies were transferred into the vial and maintained below the water line with the cotton seal. Meanwhile a timer was started to record the half-group paralysis time, which was the time when three flies failed to stand and kept lying on their back. Flies were kept at the testing temperature for 3 minutes, then transferred into a fresh vial and were left at 20 °C to recover from paralysis. The time that took the whole group to stand up was recorded as the recovery time. Five groups (n=5) were tested the same way to produce the average half-group paralysis time and recovery time. In the case of no recovery (wild-type or rescued flies), a group was observed for up to 10 minutes, and drops of water were added to the cotton seal to protect flies against dehydration.

2.3 Molecular biology

2.3.1 Extraction of Drosophila genomic DNA

Genomic DNA was prepared for cloning of endogenous Drosophila genes that were used in subsequent molecular construction of transgenic genes of fluorescent fusion proteins. The following protocol for extraction of Drosophila genomic DNA was obtained from Dr. Zhi-Chun Lai’s lab (PennState University, State College, PA). 1 to 10 flies of the same genotype were anesthetized with CO2 gas and transferred into a 1.5 ml eppendorf tube sitting on ice. 100 μl (for extraction from 1-5 flies) or 200 μl (for extraction from 6-10 flies) solution A (0.1 M Tris-Cl, 0.1 M EDTA and 1% SDS) was added to the tube, and a plastic pestle was used to homogenize the flies repeatedly until all big tissue pieces were grinded up. After the homogenate was incubated at 70 °C for 20 minutes, 8M potassium acetate was added to it at the concentration of 7 μl for every 100 μl homogenate, and was mixed by several gentle inversions of the tube. Next, the tube was kept on ice for 30 minutes and then centrifuged at 14,000 rmps for 15 minutes at 4°C. After that, the supernatant (100 μl for 1-5 flies and 200 μl for 6-10 flies) was transferred into a fresh 1.5 ml eppendorf tube and mixed with isopropyl alcohol of 1/2 of its volume (50 μl for 1-5 flies and 100 μl for 6-10 flies) at the room temperature. To precipitate genomic DNA, the tube was centrifuged at 14,000 rpm for 10 minutes at the room temperature, and the DNA pellet was then washed with 200 μl 70% ethanol and air dried in a 37 °C incubator for about 10 minutes. The dried pallet was dissolved in 18MΩ Milli Q water (5 μl for 1-3 flies, 10 μl for 4-6 flies and 15 μl for 7-10 flies). Normally, the resultant DNA solution was diluted 100 times before using as the template in polymerase chain reactions.

2.3.2 Polymerase chain reaction (PCR)

Hot start PCR was performed as described below. For each reaction, a 5 μl enzyme solution was prepared by mixing 1 μl DNA polymerase with 0.5 μl 10X DNA polymerase buffer and
3.5 μl 18 mΩ Milli Q water, and kept on ice until use. A 45 μl reaction solution was prepared by mixing together 2 μl 5 μM forward primer, 2 μl 5 μM reverse primer, 2 μl 5 mM dNTPs (5 mM dATP, 5 mM dTTP, 5 mM dCTP and 5 mM dGTP), 4.5 μl 10X DNA polymerase buffer, 2 μl DNA template and 32.5 μl 18 mΩ Milli Q water. For a negative control experiment, 2 μl 18 mΩ Milli Q water was added instead of the DNA template when making the reaction solution. After that, a few drops of mineral oil were added on top of the solution to prevent vaporization. Then the tube of reaction solution was inserted into the heat block of a Robocyclor gradient 40 PCR machine (Stratagene, La Jolla, CA) which was programmed to proceed with the following reaction steps: 1) Template denaturation at 94°C for 1 minute; 2) Primer annealing at a primer-specific temperature for 1 minute; 3) DNA chain elongation at 72 °C for calculated time duration (depends on the length of amplified sequence and the polymerase’s rate of DNA synthesis). Typically, the above steps were repeated for 30-35 cycles. For hot starting, the machine paused after step 1 in the first cycle, and then proceeded after the 5 μl enzyme solution was added to the bottom of the tube (beneath the oil layer) and mixed with reaction solution by repeated pipetting. After the last cycle, temperature was maintained at 72 °C for extra 6 minutes for the enzyme to finish all possible synthesis. Then the reaction mixture was stored at 4 °C for further analysis.

Two types of DNA polymerase were used. Specifically, for DNA analysis purpose, Taq DNA polymerase (PGC Scientific, Gaithersburg, MD) with an elongation speed of 1000bp/min was used, whereas high fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA) with an elongation speed of 500bp/min was used for DNA cloning.

### 2.3.3 Generation of DNA constructs carrying EGFP or tdTomato

Open reading frames (ORF) of endogenous genes were PCR amplified using purified genomic DNA as templates, and ORFs of EGFP and tdTomato were PCR amplified using previously generated constructs as templates. PCR primers were designed to introduce proper restriction sites to cloned ORFs and also to disrupt stop codons of ORFs designated to be on the N-terminus of fused product. ORFs were first inserted into the pBlueScript II SK vector (Fermentas INC, Canada) using the multiple cloning sites. At the final step, fused ORFs encoding fluorescent fusion proteins were shuttered into the p-element based transformation vector PUAST (Brand and Perrimon, 1993).

### 2.4 Drosophila genetics

#### 2.4.1 Generation of transgenic lines carrying GFP or tdTomato tagged synaptic proteins

Generation of transgenic fly lines was performed as described previously (Karess and Rubin, 1984; Kawasaki et al., 2002). A few hundred w^{176} flies were caged on an egg laying plate (4 mm high, 90 ml unsuphured molasses, 22 g select agar, 250 μl tegasept stock and 556 ml distilled H2O) and trained for a few days before eggs were colleted for DNA injection. Young embryos (eggs freshly laid within 30 minutes) were harvested from the plate and aligned in a row of 20-25 on surface of a 2% agar cube (20×13×5mm). The anterior end of embryos was orientated towards the edge of the cube. Then embryos were glued (glue was made by dissolving double sticky tapes in Heptane) onto surface of a glass cover slip (18×18mm,
VWR, West Chester, PA), which was then placed on top of a micro slide glass (25×75mm, VWR, West Chester, PA). After that, a strip of oil (27 Halocarbon oil and 700 Halocarbon oil mixed at 1:19, Sigma, St. Louis, MO) was applied to cover all the embryos.

Plasmid carrying the transformation construct was prepared using the Qiagen Midiprep kit (Qiagen, Valencia, CA). To prepare the injection solution, transformation plasmid was mixed with helper plasmid (p_25.7wc, carrying the p-element transposase gene), green food color (McCormick) and injection buffer (50 mM KCl, 1 mM NaH₂PO₄ and 1 mM Na₂HPO₄, pH 6.8). The final concentrations of each ingredient were as follows: Transformation plasmid: 0.4 to 0.8 μg/μl; Helper plasmid: 0.2 μg/μl; Green food color: 1:10 diluted; Injection buffer: 1:10 diluted. Injection needles were made by pulling glass capillary tube with an electrode puller (Sutter, Novato, CA). Each needle was loaded with 1 μl or so injection solution.

Microinjection was performed on an upright microscope (Zeiss, Germany). The micro slide glass carrying glued young embryos was placed under the microscope with the posterior end of embryos facing the needle. A micro-manipulator was used to control the movement of needle. DNA was injected into the posterior tip of young embryos before cellularization. After injection, cover slips carrying glued embryos were placed on top of fly food in a plastic plate (100mm in diameter and 13mm in height). Food plates each containing 6-8 cover slips (120-200 embryos) were left at the room temperature for embryos to hatch.

Hatched F0 progeny were transferred into fresh food vials (roughly 100 larvae per vial) within 2-3 days after injection, and then crossed to flies with w background. The w⁺ marker carried by pUAST was used to select transformed progeny. A detailed crossing scheme of establishing homozygous transgenic stocks is provided in Figure 2.1.
Figure 2.1 Crossing Scheme of Establishing Homozygous Transgenic Stocks
2.4.2 Screening for transgenic lines

Transgenic expression of fluorescent fusion protein through the UAS-GAL4 system has position effect. That is, expression level of a transgene is affected by its inserted location on a chromosome. Therefore, transgenic fly lines of different expression levels were screened with an imaging-based procedure. For each line, males homozygous for the transgenic insertion were crossed to Apal-GAL4 females. Male (for autosomal insertions) or female (for X chromosomal insertions) F1 progeny were dissected at the third-instar larval stage, and ventral longitudinal body wall muscles 6 and 7 within in abdominal segment A2 or A3 were imaged with an epifluorescence imaging system. A set of (typically 3) images captured with different exposure times were taken from each hemi-segment, and typically, totally 4 hemi-segments from 2 dissected larvae were imaged for each transgenic line. Using these images, fluorescence intensity within synaptic terminals and brightness of background autofluorescence of each line were categorized as High, Medium or Low levels. Therefore, transgenic lines of varied expression level were selected according to different experimental purposes based on these categorizations. Also, lines of low background autofluorescence were selected to establish a preferable signal-to-noise ratio during imaging analysis.
A. Innervation of larval peripheral body wall muscle fibers

B. Epifluorescence image of nerve terminals expressing soluble EGFP

Figure 2.2 *Drosophila Larval neuromuscular system.* A) Several of the identified motoneuron and muscle fibers of an abdominal hemisegment are shown. Muscles are shown as overlapping layers of striated fibers. Ventral longitudinal muscle 6 and 7 are big fibers located on the most inner surface of the body wall, and are innervated by two motor neurons with synapses formed on surfaces (shown in blue). Adapted from Keshishian and Chiba (1993). B) Epifluorescence image of nerve terminals expressing soluble EGFP. Nerve terminals (fine branches and boutons) light in green by EGFP fluorescence are located on surfaces of ventral longitudinal muscle 6 and 7, shown as thick grey strips on right and left respectively.
2.5 Biochemistry

2.5.1 Co-immunoprecipitation

Co-immunoprecipitation was performed following the protocol adapted from Irwin Levitan (University of Pennsylvania, Philadelphia, PA) with several modifications. To investigate co-immunoprecipitation of other components of the 20S fusion particle with SYX, active wild-type (Canton S) and comt<sup>ST17</sup> mutant flies were transferred into separate 15 ml centrifugation tubes (VWR, West Chester, PA), and completely immersed into a 38 °C water bath. After a 10-minute exposure to 38 °C, tubes were immediately immersed in liquid nitrogen. Next, the tubes went through multiple (at least 3) rounds of vortexing and re-freezing to decapitate frozen flies. Fly heads were separated from other body piece by 2-step sieving with 0.71 and 0.355 mm opening sieves (Fisher Scientific, Pittsburgh, PA) at the room temperature and weighed on a scale. Then certain amounts of heads were transferred into 1.5 ml eppendorf tubes sitting on ice. Approximately 600 heads (~ 60 mg) can be recovered from 750 flies. Fresh ice-cold lysis buffer [1% CHAPS, 20 mM Tris/HCl (pH 7.5), 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 2 mM DTT (dithiothreitol), 1:100 protease inhibitor cocktail (Sigma St. Louis, MO)] was added to tubes at the concentration of 1 head/μl, and heads were homogenized with a Teflon pestle. To prevent protein degradation, tubes were placed on ice for 30 seconds after every 30-second homogenization. To achieve maximal grinding, cell debris was pelleted by centrifugation at 9,000 g for 5 minutes and then homogenized once again. The homogenized sample was incubated for 30 minutes with constant agitation and then centrifuged at 9,000 g for 10 minutes to produce the final head lysate (supernatant).

Protein-A-sepharose beads (Amersham Biosciences, Arlington Heights, IL) were washed 3 times before using. For each wash, 25 μl beads were mixed with 1.5 ml fresh ice-cold lysis buffer in a 1.5 ml eppendorf tube by flicking and inverting the tube several times, and then beads were recovered by centrifugation at 1,300 g for 1 minute. After wash, beads with or without BSA blocking were made into a 50% slurry by mixing 25 μl beads with 25 μl fresh ice-cold lysis buffer. For BSA blocking, 25 μl washed beads were mixed with 400 μg high quality BSA and 1 ml fresh ice-cold lysis buffer, incubated with constant agitation for 10 minutes and recovered by centrifugation at 1,300 g for 1 minute.

To perform immunoprecipitation, 550 μl head lysate was transferred into a fresh 1.5 ml eppendorf tube using a 200 μl pipet, and precleared with 50 μl of a 50% slurry of unblocked beads for 1 hour with constant agitation. After pre-clearing, beads were pelleted by centrifugation at 1,300g for 1 minute, and 20 μl of the pre-cleared lysate was saved as pre-IP input and boiled for 3 minutes before gel-loading. 500 μl of the remaining lysate was mixed with 50 μl of a 50% slurry of BSA blocked beads and 5μg concentrated mouse anti-SYX antibody (mAb 8C3) or equivalent amount of pre-immune mouse IgG (as a negative control group) and incubated for 2 hours with constant agitation. After this step, beads were pelleted with centrifugation at 1,300g for 1 minute and washed for 5 times. For each wash, beads were suspended in 1 ml fresh ice-cold lysis buffer and mixed with constant agitation for 5 minute and recovered with centrifugation at 1,300 g for 1 minute. To elute associated complexes, beads were resuspended in 15 μl SDS sample buffer [125 mM Tris/HCl/SDS, pH 6.8, 2%...
(w/v) SDS, 1% 2-mercaptoethanol, 10% (v/v) glycerol and 0.5% Bromophenol Blue] and boiled for 3 min. All centrifugation and incubation procedures were carried out at 4 °C.

2.5.2 Western blotting
Conventional western-blot analysis was performed following the immunoprecipitation procedure as described previously (Kawasaki, et al, 2004). For analysis of pre-IP inputs and IP samples, the equivalent of 0.5 head and 400 heads respectively was loaded per lane onto a 9% SDS-PAGE gel. 2 μl of dual size standard (Bio-Rad, Hercules, CA), was loaded in lane next to the samples for protein size estimation. The gel was run in SDS electrophoresis buffer (25 mM Tris, 190 mM glycine and 1% SDS) with a constant current of 15mA per gel at the room temperature for 1 hour. After electrophoresis, proteins were wet transferred onto nitrocellulose membranes (Pall, Port Washington, NY) in transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol) with a constant voltage of 14 volts. Protein transferring was carried out overnight at the room temperature with an ice cube kept next to the transfer cassette according to the manufacturer’s direction (Bio-Rad, Hercules, CA). Next, membranes were blocked with 5% non-fat milk in PBS (Brown’s Lab, 171 mM NaCl, 4 mM Na2HPO4, 3.4 mM KCl, and 1.84 mM KH2PO4, pH 7.5, all incubations were carried out at the room temperature unless specified) for 1 hour, then incubated with primary antibody diluted in 5% milk at 4 °C with constant agitation overnight. After primary antibody incubation, membranes were washed in PBST solution (0.1% Tween-20 in PBS) 3 times for 10 minutes each, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:5,000 in 5% milk with constant agitation for 2 hours. To remove unbound secondary antibodies, membranes were washed again in PBST solution 3 times for 10 minutes each. Detection was accomplished using enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Arlington Heights, IL). Solution A and B were mixed at the ratio of 40:1 and applied to membranes to react in dark for 5 minutes at the room temperature. After reaction, membranes were immediately scanned with a phosphor image scanner (GE Healthcare, Piscataway, NJ) and then exposed to X-ray films (Amersham Biosciences, Arlington Heights, IL). To re-blot with a second primary antibody, membranes were washed once in PBST and then twice in PBS, 10 minutes each, and then blocked again in 5% milk for 1 hour.

2.5.3 Immunocytochemistry
Immunocytochemistry of Drosophila larval neuromuscular synapses was performed as described previously (Kawasaki et al., 2004) with several modifications. Drosophila third-instar larvae were dissected in saline solution (128 mM NaCl, 2 mM KCl, 1.8 mM Ca2+, 4 mM Mg2+, 5 mM Hepes and 36 mM sucrose, pH 7.0) at 20 °C as described in 2.6.1. Preparations were then fixed for 30 minutes (all incubations were carried out at the room temperature unless specified) in saline solution containing 4% paraformaldehyde. To investigate activity-dependent redistribution of dNSF1, specifically, in experiments shown in Figure 3.2 B and C, dissected specimen were perfused with saline solution at 33 °C for 2 minutes and then subjected to 1 minute of 20 Hz stimulation followed by immediate fixation with warm (33 °C) fixative (4% paraformaldehyde in saline solution). After fixation, preparation were treated with consecutive 10-minute washes with saline solution, PBS, and PBT (0.2% Triton X-100 in PBS) respectively, then preparations were incubated with
blocking buffer (5% normal goat serum in PBT) for 1 hour, and then with primary antibodies
diluted in blocking buffer at 4 °C overnight. Next, preparations were washed three times with
PBT then twice with PBS for 6 minutes each. Washed preparations were incubated with
fluorescent-conjugated secondary antibodies diluted in PBS containing 5% normal goat
serum for 2 hours. After secondary antibody incubation, preparations were washed five times
with PBS for 6 minutes each and mounted between cover slips (Corning, Corning, NY) with
a 1:1 mixture of PBS and glycerol for imaging.

2.5.4 Antibodies

Co-immunoprecipitation:
For immunoprecipitation of SYX, 5μg concentrated mouse anti-SYX antibody, mAb 8C3
(DSHB Hybridoma, University of Iowa, IO), were added to reaction mixture containing 500
μl pre-clear head lysate and 50 μl of 50% slurry of BSA-blocked Protein-A sepharose beads.
For negative control group, 5μg pre-immune mouse IgG (Jackson ImmunoResearch
Laboratories, West Grove, PA) was added instead of mAb 8C3.

Western blotting:
For primary antibody incubation, a mouse anti-SYX antibody (crude serum), mAb 8C3
(DSHB Hybridoma, University of Iowa, IO), was used at a dilution of 1:10. A rabbit
anti-nsyb antibody, developed in Dr. Richard Ordway’s lab (PennState University, State
College, PA), was used at a dilution of 1:30. A rabbit antibody recognizing the epitope at the
N-terminus of SNAP-25, a gift from Dr. David Deitcher (Cornell University, Ithaca, NY),
was used at a dilution of 1:2,000. Rabbit anti-dNSF and anti-SNAP antibodies, kindly
provided by Dr. Leo Pallanck (University of Washington, Seattle, WA), were used at a
dilution of 1:500 and 1:1,000 respectively. For secondary antibody incubation, horseradish
peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies (Amersham
biosciences, Arlington Heights, IL) were used at a dilution of 1: 5,000.

Immunocytochemistry:
For primary antibody incubation, a rabbit anti-dNSF antibody, kindly provided by Dr. Leo
Pallanck (University of Washington, Seattle, WA) was used at the dilution of 1:5,000. A
mouse anti-CSP antibody, kindly provided by Dr. Konrad Zinsmaier (University of Arizona,
Tucson, AZ) was used at a dilution of 1:200. And a mouse anti-BRP antibody (mAb nc82),
kindly provided by Dr. Erich Buchner (University of Wurzburg, Germany) was used at a
dilution of 1:50. For secondary antibody incubation, fluorescent-conjugated secondary
antibodies, Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-mouse antibodies
were used at a dilution of 1:200 (Invitrogen, Carlsbad, CA). To visualize neuronal plasma
membranes, a Cy5-conjugated rabbit anti-HRP (horseradish peroxidase) antibody (Jackson
ImmunoResearch Laboratories, West Grove, PA) was used at a 1:200 dilution during
secondary antibody incubation. (Sun and Salvaterra, 1995).
2.6 Microdissection and preparation

2.6.1 Larval preparation

A wondering third-instar larva was selected and transferred with forceps into a dissection chamber. The larva was washed twice with 18 mΩ Milli Q water then once with dissection saline solution (128 mM NaCl, 2 mM KCl, 1.8 mM Ca\(^{2+}\), 4 mM Mg\(^{2+}\), 5 mM Hepes and 36 mM sucrose, pH 7.0). To dissect, the larva was stretched out lengthways and pinned down on its abdomen with a pair of minutien pins which were placed next to its mouse hook and between its posterior spiracles respectively. After a small incision was made on its dorsal midline with spring scissors, the larva was covered with dissection solution. After that, one blade of the scissors was inserted into the incision, and the cuticle was cut along the dorsal midline first towards the rostral pin then towards the caudal pin. After cutting, internal tissues floated up out of the body were removed carefully with forceps. Then the left and right flaps of cuticle were stretched open sidewise and pinned down symmetrically with 3 pins on each side near the edge of cut. Positions of pins were adjusted to ensure symmetrical stretching of the cuticle in both horizontal and vertical directions. Next, the tracheal system and organs were removed as completely as possible with forceps, leaving the ventral ganglion, nerves and body wall muscles intact (Figure 2.2). For live-imaging or immunocytochemical analysis of synaptic terminals, the ventral ganglion was also removed after all nerves projecting from it were cut. To get rid of floating tissue pieces, the chamber was washed with fresh saline solution as needed during dissection.
Figure 2.3 Dissected *Drosophila* 3\textsuperscript{rd} instar larval preparation. A wondering 3\textsuperscript{rd} instar larva was pinned down on its abdomen with minutien pins placed at its anterior and posterior ends. Cuticle was cut along the ventral midline, and pinned down symmetrically on both sides with minutein pins. Soft tissues and tracheae were removed completely to expose the ventral ganglion, nerves and crossbedded peripheral body wall muscle fibers.
2.6.2 Adult dorsal longitudinal flight muscle (DLM) preparation

Dissection of adult *Drosophila* was performed as previously described (Kawasaki, 1998). Male or female fly of 3-5 days age was anesthetized with CO₂ gas, and then mounted laterally with wax over a hole of an air tube as described (Koenig *et al.* 1989). Fly was then dissected in saline solution (128 mM NaCl, 2 mM KCl, 1.8 mM Ca²⁺, 4 mM Mg²⁺, 5 mM Hepes and 36 mM sucrose, pH 7.0), with air delivered to the tracheal system through its underside (mounted under wax) using an aquarium pump. The lateral surface of the dorsal longitudinal flight muscle (DLM), which contains 6 identifiable fibers and the thoracic ganglion of the CNS were exposed by dissection (Figure 2.3). To eliminate evoked synaptic activity and prevent muscle contraction during live-imaging, neck and posterior dorsal mesothoracic nerve (PDMN) projecting from the thoracic ganglion were both cut. In Figure 2.3, DLM fibers were shown diagrammatically as white stripes within the thorax (left) and in an image of a dissected DLM preparation (right).

![Figure 2.4 Illustration of *Drosophila* dorsal longitudinal flight muscles (DLMs). DLM are shown diagrammatically as white stripes within the thorax (left) and in an image of a dissected DLM preparation (right).](image_url)
2.7 Imaging

2.7.1 Epifluorescence microscopy

An epifluorescence imaging system was used to perform the imaging-based screening of transgenic fly lines as described in 2.4.2. Third-instar larvae were dissected as described in 2.6.1. Ventral longitudinal body wall muscles 6 and 7 within abdominal segment A2 or A3 of dissected larval preparation were imaged with a Nikon (Tokyo, Japan) Eclipse E600FN microscope equipped with a Fluor 60x 1.0 numerical aperture water-immersion objective (Nikon) and a DG-5 175 watt Xenon lamp (Sutter, Novato, CA). Autofluorescence images were captured with a CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan). EGFP was imaged with the following filter set: excitation filter, HQ480/20; dichroic mirror, Q495LP; emission filter, HQ 525/50 (Chroma, Brattleboro, VT). And DsRed or tdTomato were imaged with the following filter set: excitation filter, HQ545/30; Dichroic mirror Q570LP; emission filter, HQ620/60 (Chroma, Brattleboro, VT). Images were acquired with the Meta Imaging Series 6.1 software package (Universal Imaging Corporation, Downingtown, PA) with 100% excitation, binning of 2x2 binning, offset of 0, gain of 1, and specified exposure times. From each hemisegment, typically a set of 3 images were taken with different exposure times, for example, 1s, 2s and 5s, for a better comparison of signal or background fluorescence intensity among lines.

2.7.2 Confocal microscopy

For immunocytochemistry, mounted preparations were analyzed with the Olympus FV1000 confocal imaging system (Tokyo, Japan) available at the Fluorescence Imaging Facility of the Huck Institute at Pennsylvania State University. All preparations were imaged under a PlanApo 60x 1.4 numerical aperture oil objective (Olympus, Japan). Sequential XYZ scanning images were acquired with gain of 1, offset of 0, C.A (confocal aperture) of 2, zoom of 5 and at a z-step size of 0.2μm. Image acquisition and processing were performed using the FluoView software package (Olympus, Japan). After processing, maximum projections of two optical sections were generated for a display.

2.7.3 Live-imaging

2.7.3.1 Live preparation and imaging

A Third-instar larva was dissected as described in 2.6.1. The ventral ganglion was removed after all nerves projecting from it were cut near muscles. After that, the dissection chamber was perfused with saline solution (128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 1.8 mM CaCl₂, 36 mM sucrose, and 5 mM HEPES, pH, 7.0) at the rate of 0.5 ml/min. And the incubation temperature was maintained at 20 °C using a Medical Systems TC-202 temperature controller and PDMI micro-incubator (Medical Systems, Greenvale, NY). One nerve that innervates abdominal hemisegment A2 or A3 was pulled into a suction electrode filled with saline solution for stimulation. A Master-8 stimulator (A.M.P.I, Jerusalem, Israel) was used to generate electrical pulses within the nerve. After a single 1.5 v stimulus was delivered, responding muscle contraction was observed as an indicator of successful preparation and stimulation. Then the preparation was perfused with saline solution containing 10
L-glutamic acid and 20 μM nicardipine to prevent random muscle movement or contraction responding to evoked transmitter release. Experiments normally began at 20~30 minutes after drug perfusion started. With the perfusion rate at 0.5 ml/min, temperature shifts from 20 to 33°C usually required 4 min.

Live-imaging was performed using a Zeiss LSM confocal microscope (Carl Zeiss, Germany) equipped with a Plan-Apochromat 63x water immersion objective (Carl Zeiss, Germany), an Ar 488 nm laser and a He-Ne 543 nm laser. EGFP was excited with 488 nm laser line and emission was collected through a Z488/543RPC dichroic mirror and a HQ520/40 filter (Chroma, Brattleboro, VT). Image was acquired using the Carl Zeiss LSM software Version 3.99 with 0.1% laser power, zoom of 4, pinhole size of 100, contrast of 9950 and varied PMT values.

2.7.3.2 Fluorescence recovery after photobleaching (FRAP)

To investigate in vivo mobility of dNSF1 and SNAP at native synapses, neuromuscular synapses expressing dNSF1-EGFP and EGFP-dSNAP was analyzed by fluorescence recovery after photobleaching (FRAP). A single bouton of ventral longitudinal muscle 6 and 7 within abdominal segment A2 or A3 was analyzed in each experiment. One frame (Frame 0) of image was taken at the bouton prior to photobleaching. To generate a bleached region within the bouton, horizontal line scanning across the center of bouton was performed with maximum 488 nm laser power for 10 times. Immediately after that, a continuous time-lapse series of 128 frames was taken at the rate of 0.469s/frame to monitor FRAP within the bouton for 1 min.

2.7.3.3 Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) was employed to investigate in vivo interactions among synaptic proteins, including SNAREs, dNSF1 and SNAP. FRET imaging was also carried out with the confocal live-imaging system. Detailed imaging methodologies and paradigms of two FRET detection methods, the 3-Cube FRET and donor dequenching measurements, will be discussed in Chapter 4.

2.8 Data analysis

Imaging processing was carried out with the Meta Imaging Series 6.1 software package (Universal Imaging Corporation, Downingtown, PA). Numerical data analysis and graphing were performed with Microsoft Excel (Seattle, WA). All data values in the text and bar graphs were given as mean ± SEM. Statistical analysis was performed using the two-tailed Student’s t test, and three significance levels were assigned to be p≤0.05, p≤0.01 and p≤0.001 (indicated as a single, double or triple asters in graphs).

2.8.1 Quantification of activity-dependent redistribution

Background subtraction, photobleaching correction and image subtraction were carried out using the Arithmetic function of the Meta Imaging Series 6.1 software (Universal Imaging Corporation, Universal Imaging Corporation, Downingtown, PA). Curve-fitting of histograms
was performed using the data analysis program IGOR (WaveMetrics, Lake Oswego, OR).

2.8.2 FRAP

To analyze the data, two regions of interest (ROIs) were drawn to closely surround the bouton (later referred to as the ‘whole bouton’) and the bleached region (later referred to as the ‘bleached region’) respectively. Both regions were then duplicated and placed about 3μm away (horizontally) to measure the background due to autofluorescence. Region measurements of integrated fluorescence intensity were logged as numerical data into Microsoft Excel (Seattle, WA). Background was then subtracted from the ROI fluorescence. After background subtraction, fluorescence (within both ROIs) of Frame 1-128 was normalized individually to Frame 0 (taken prior to photobleaching). Normalized whole bouton fluorescence was plotted against the frame number (N). The curve was fitted with a linear trendline, of which the slope (k) was used to correct for undesired photobleaching due to image acquisition. For each frame (frame N), |k|×N was added to normalize fluorescence (within both ROIs). Finally fluorescence recovery within the bleached region was scaled and plotted as a function of time. For scaling, 100% recovery was defined as the average of normalized whole bouton fluorescence of Frame 1-128, and the 0% recovery was defined as the normalized bleached region fluorescence of Frame 1.

2.8.3 FRET

3-Cube FRET images were taken at individual presynaptic boutons. Image processing including ROI drawing and background subtraction were performed in the same way as described for FRAP. A FRET ratio (FR) was calculated for each bouton as described below.

For analyzing resting synapses, multiple boutons were sampled at random time points. Data of high temperature experiments included boutons sampled at no longer than 25min after the 33 °C was reached. The mean FR represented the average of minimum 16 boutons from 4-5 larval preparations. For analyzing stimulated synapses, one bouton was sampled at specific time points before, during and after stimulation in each experiment. The mean FR (calculated for each sampling time point) represented the average of minimum 6 independent experiments.

Data analysis for the 3-Cube FRET essay was adapted from previous publications (Erickson et al., 2001; Erickson et al., 2003). Briefly, three constant values, $R_A$, $R_{D1}$ and $R_{D2}$, defining the ratios of emission between cubes ($S_{cube(specimen)}$), can be experimentally determined by the following equations, when the donor (D) or acceptor (A) was expressed solely in the system:

\[
R_A = \frac{S_{\text{FRET}(A)}}{S_{\text{tdTomato}(A)}} \\
R_{D1} = \frac{S_{\text{FRET}(D)}}{S_{\text{EGFP}(D)}} \\
R_{D2} = \frac{S_{\text{tdTomato}(D)}}{S_{\text{EGFP}(D)}}
\]

These constant values were used to correct for channel cross-talk due to spectra overlapping of the donor and acceptor fluorophores. Finally, when both fluorophores were co-expressed (DA), the FRET ratio (FR) reflecting the acceptor fluorescence intensity increase due to
FRET was calculated as:

\[ FR = \frac{F_{AD}}{F_A} = \frac{(S_{FRET} - S_{EGFP} \times R_{D1})}{[(S_{tdTomato} - S_{EGFP} \times R_{D2}) \times R_A]} \]

Note that \( FR > 1 \) indicates FRET whereas \( FR = 1 \) indicates no FRET.

Detailed data analysis for 3-Cube FRET and a second FRET detection method, the donor dequenching measurement, will be discussed in Chapter 4.
Chapter 3  *In vivo* functions and interactions of NSF and SNAP in synaptic vesicle trafficking and neurotransmitter release

3.1 Introduction

Previous studies of the neurotransmitter release apparatus have identified a highly conserved core of interacting proteins including: N-ETHYLMALEIMIDE SENSITIVE FACTOR (NSF), the SOLUBLE NSF ATTACHMENT PROTEIN (SNAPs) and the SNAP RECEPTORS (SNARES) (Söllner *et al*., 1993). Current models suggest that one synaptic vesicle membrane SNARE protein (v-SNARE), SYNAPTOBREVIN (SYB), and two presynaptic plasma membrane SNAREs (t-SNAREs), SYNTAXIN (SYX) and SYNAPTOSOME ASSOCIATED PROTEIN of 25-kDa (SNAP-25), assemble into a ternary SNARE complex which promotes synaptic vesicle fusion and neurotransmitter release (Jahn and Scheller, 2006). Full vesicle fusion produces cis-SNARE complexes in the plasma membrane which are subsequently disassembled by NSF and SNAPs. After complex disassembly, free t-SNAREs are available to participate in new priming reactions, whereas v-SNAREs are incorporated into recycled vesicles through a dynamin-dependent endocytotic process.

Despite great progress in defining molecular interactions of NSF, SNAPs and SNAREs, it is critically important to better connect abundant biochemical data with the *in vivo* functions and interactions of these proteins in neurotransmitter release. An exciting opportunity to meet this challenge is provided by the *Drosophila* genetic model system, in which conserved mechanisms of neurotransmitter release may be investigated *in vivo* using a powerful combination of genetic, molecular, electrophysiological and imaging methods. The current study has employed live imaging of NSF and SNAP fluorescent fusion proteins and the temperature-sensitive (TS) paralytic dNSF1 mutant, *comatose* (Siddiqi and Benzer, 1976; Ordway *et al*., 1994; Pallanck *et al*., 1995a), to examine the *in vivo* behavior and interactions of dNSF1 and SNAP at native synapses.

3.2 Results

3.2.1 Accumulation of protein complexes containing SNAREs, dNSF1 and SNAP upon inactivation of dNSF1

The *Drosophila* NSF protein, dNSF1, plays an important role in synaptic vesicle trafficking and neurotransmitter release (Pallanck *et al*., 1995a; Kawasaki *et al*., 1998; Littleton *et al*., 1998; Kawasaki and Ordway, 1999, 2009). dNSF1 is thought to disassemble plasma membrane SNARE complexes (presumably *cis*-SNARE complexes) on the basis of biochemical analysis in *comatose* mutants showing plasma membrane accumulation of SDS-resistant *cis*-SNARE complexes following disruption of dNSF1 activity (Littleton *et al*., 1998; Tolar and Pallanck, 1998). As a first step in examining the *in vivo* interactions of dNSF1 and dSNAP with SNARE complexes, co-immunoprecipitation studies (co-IP) were carried out in *comatose* to investigate whether accumulated SNARE complexes were associated with dNSF1 and SNAP. IP of *Drosophila* SYX 1A from *Drosophila* head homogenates and subsequent western analysis was performed to examine co-IP of NSYB, SNAP-25, dNSF1 and SNAP in head homogenates from wild-type and *comatose* flies exposed to a restrictive temperature of 38 °C for 10 minutes (Figure 3.1). NSYB, SNAP-25,
dNSF1 and SNAP were found to co-IP with SYX 1A, consistent with previous studies demonstrating assembly of SNAREs, NSF and SNAPs into a 20S protein complex (Wilson et al., 1992; Söllner et al., 1993a). While a similar amount of SYX 1A IP was observed in comatose and wild-type samples (Figure 3.1 A), co-IP of each protein was consistently higher in comatose (Figure 3.1 B) indicating that acute disruption of dNSF1 activity leads to accumulation of (20S) protein complexes containing dNSF1, dSNAP and SNAREs. These results are consistent with in vitro studies demonstrating that inhibition of NSF activity arrests disassembly of 20S particles containing NSF, SNAP and SNAREs.

![Figure 3.1 Co-immunoprecipitation of endogenous core complexes containing dNSF1, SNAP and SNAREs. Canton S (CS) and comatose flies were exposed to 38 °C for 10 minutes before decapitation, and head homogenates were prepared as described in 2.5.1. A monoclonal SYX antibody (mAb 8C3) was used to pull down endogenous SYX from the fly head homogenates. A negative control was included to illustrate the specificity of IP, in which equivalent amount of pre-immune mouse IgG was added instead of mAb 8C3. Also, pre-IP inputs (see 2.5.1) were loaded on gel to demonstrate the presence of equal amounts of target proteins in each sample before the IP procedure.) Pre-IP inputs and IP samples were blotted for SYX, NSYB, SNAP-25, dNSF1 and SNAP. Note the presence of similar amounts of target proteins in each pre-IP input. a) Specific IP of SYX from CS and comatose samples incubated with anti-SYX IgG. Note the failure of IP from the CS sample incubated with mouse IgG. b) Co-IP of NSYB, SNAP-25, dNSF1 and SNAP. Note the elevated amounts of co-IP from the comatose sample.
3.2.2 Presynaptic localization of endogenous dNSF1 at native synapse

To help place the preceding interactions of dNSF1 and SNAP in the context of native synapses, the in vivo distribution of dNSF1 was examined using the larval neuromuscular synapse as a model. As shown in Figure 3.2 A, immunolabeling of wild-type preparations with a polyclonal anti-dNSF antibody revealed a dNSF1 distribution similar to that of synaptic vesicles, which occupy cytosolic regions within presynaptic boutons. This dNSF signal was eliminated in the dNSF1 null mutant, \( \text{comt}^{\text{CLP1}} \) ((Golby et al., 2001; Sanyal and Krishnan, 2001), demonstrating that it specifically reflects the distribution of dNSF1 (Figure 3.31). These findings indicate dNSF1 is localized primarily to the cytosol of presynaptic boutons.

3.2.3 dNSF1 redistributes to periactive zone regions of the presynaptic plasma membrane in comatose

To examine the in vivo distribution of dNSF1 under conditions favoring its assembly with SNAP and SNARE proteins, dNSF1 localization was examined at comatose larval neuromuscular synapses as well. Wild-type and comatose preparations at the restrictive temperature of 33 °C were stimulated at 20 Hz for 1 minute followed by immediate chemical fixation and subsequent processing for immunocytochemistry. These experiments revealed a striking redistribution of dNSF1 at comatose, but not wild-type, neuromuscular synapses (Figure 3.2 B,C). Localization of dNSF1 was determined relative to markers for synaptic vesicles (Figure 3.2 B) as well as active zone (AZ) regions of the presynaptic plasma membrane where synaptic vesicles dock and fuse (Figure 3.2 C). Following its redistribution in comatose, dNSF1 exhibited a punctuate pattern which no longer resembled that of a synaptic vesicle marker (Figure 3.2 B). Moreover, a clear relationship was observed between the punctuate pattern of redistributed dNSF1 and an active zone marker. dNSF1 puncta were adjacent to active zones such that the active zone marker fit nicely into dim regions of the dNSF1 distribution (Figure 3.2 u-y). In contrast, the dNSF1 distribution at wild-type synapses remained similar to that of a synaptic vesicle marker (Figure 3.2 f-j) and exhibited no clear relationship to active zones (Figure 3.2 p-t). The preceding findings indicate that disruption of dNSF1 function in comatose results in its redistribution to PAZ regions of the presynaptic plasma membrane.
Figure 3.2 Confocal immunofluorescence images of endogenous dNSF1 localization at larval neuromuscular synapses. A) Labeling with anti-HRP, anti-dNSF and anti-Cysteine String Protein (CSP) as a marker for synaptic vesicles in WT at 20 °C. B, C) Redistribution of endogenous dNSF1 to PAZ regions of the plasma membrane in comatose at the restrictive temperature of 33 °C. WT and comatose synapses were subjected to 20 Hz stimulation (stim) for 1 min followed by chemical fixation and processing for immunocytochemistry. To include an active zone marker, a monoclonal antibody (mAb nc82) was used to label the Drosophila Bruchpilot protein (BRP), an identified active zone T-bar component (Wagh et al., 2006). Labeling with anti-HRP, anti-dNSF and anti-CSP (B), or anti-BRP as a marker for presynaptic active zones (C). All images represent maximum projections of 2 optical sections.
3.2.4 Redistribution of dNSF1 is activity-dependent

The properties of dNSF1 redistribution in *comatose* were further examined in live imaging studies employing presynaptic expression of a GFP-tagged dNSF1 protein, dNSF1-EGFP, at larval neuromuscular synapses. For analysis in *comatose*, a TS mutant form of the tagged protein, dNSF1<sup>ST17</sup>-EGFP, was expressed in a *comatose* (*com<sup>ST17</sup>* mutant) background. This approach permits live imaging of dNSF1 before, during and after synaptic stimulation to further define the properties and activity-dependence of the dNSF1 redistribution observed in *comatose* (Figure 3.3 A). At wild-type synapses maintained at 33 °C, 1 minute of 20 Hz stimulation had little impact on dNSF1-EGFP distribution (*upper panel*). In *comatose* at 33 °C prior to stimulation, the distribution of dNSF1<sup>ST17</sup>-EGFP resembled that of dNSF1-EGFP in wild-type. However, the same stimulation paradigm at *comatose* synapses produced clear redistribution of dNSF1<sup>ST17</sup>-EGFP into a punctate pattern which persisted long after the stimulation train (*lower panel*). Finally, redistribution of dNSF1<sup>ST17</sup>-EGFP was conditional in the *comatose* mutant as it was not observed after 20 Hz stimulation at 20 °C (Figure 3.S2). Activity-dependent redistribution was quantified by comparing images taken before and during the stimulation train (after correction for photobleaching) and determining intensity differences for each pixel. Histograms of pixel intensity differences indicated substantially larger differences in *comatose* versus wild-type control synapses and these were reflected in the width of Gaussian fits (Fig 3.3 B). The respective mean values of the full width at half maximum (FWHM) for wild type and *comatose* were 9.2 ± 0.78 (n=4) and 18.2 ± 0.85 (n=4) which were significantly different (p<0.001) (Figure 3.3 C). Taken together, these results demonstrate activity-dependent redistribution of dNSF1 following disruption of dNSF1 function in *comatose*. Notably, maintaining *comatose* mutant synapses at 33 °C for an extended period (~15 minutes) led to redistribution of dNSF1<sup>ST17</sup>-EGFP in the absence of stimulation (data not shown). This observation may reflect cumulative spontaneous vesicle fusion over this time period. The preceding findings indicate activity-dependent redistribution of dNSF1 to PAZ regions of the presynaptic plasma membrane.
Figure 3.3 Activity-dependent redistribution of dNSF1 in comatose. A) Activity-dependent redistribution of dNSF1 was examined by live imaging synapses expressing wild-type dNSF1-EGFP in a wild-type background or dNSF1^{ST17}-EGFP in a comt^{ST17} background. In each case, a presynaptic bouton was imaged once at 20 °C (A), and then multiple times at indicated time points (B-E) at 33 °C. 1 minute of 20 Hz stimulation was applied after the preparation had been exposed to 33 °C for 2 minutes. B) Image D (during stimulation) was subtracted from Image C (before stimulation) after correction for photobleaching. Intensity differences for each pixel were plotted in histograms and fit with Gaussian curves. C) The respective full width at half maximum (FWHM) for wild type and comatose were 9.2 ± 0.78 (n=4) and 18.2 ± 0.85 (n=4), which were significantly different from each other (p<0.001).
3.2.5 Activity-dependent redistribution of dNSF1 results in a drastic decrease in dNSF1 mobility

As described in the preceding section, activity-dependent redistribution of dNSF1ST17-EGFP persisted long after the stimulation train. To examine whether redistributed dNSF1 is mobile or rather stably associated with the PAZ, Fluorescence Recovery After Photobleaching (FRAP) was employed to assess the mobility of dNSF1. Presynaptic boutons expressing dNSF1-EGFP (in a wild-type background) or dNSF1ST17-EGFP (in a comST17 background) were maintained at 33 °C. Prior to photobleaching, boutons were subjected to 1 minute of 20 Hz stimulation to elicit dNSF1 redistribution in comatose. Individual boutons were then photobleached across the center and fluorescence recovery within the bleached region was monitored by time-lapse imaging. Exemplary images of stimulated comatose and wild-type control boutons are shown in Figure 3.4 A. For each experiment, recovery of fluorescence intensity within the bleached region of interest (ROI) (as a percentage of maximal recovery) was plotted as a function of time (Figure 3.4 B). The mobility of wild-type dNSF1-EGFP was not affected by stimulation (black traces). In contrast, dNSF1ST17-EGFP exhibited a drastic decrease in mobility after stimulation (gray traces). These studies reveal that dNSF1ST17-EGFP is immobilized at the PAZ following its activity-dependent redistribution and suggest a strong interaction between dNSF1 and other PAZ components. Although both dNSF1-EGFP and dNSF1ST17-EGFP exhibited cytosolic distributions before stimulation, dNSF1ST17-EGFP had a significantly lower mobility (Figure 3.4 B left). This discrepancy may be explained by enhanced binding between dNSF1 and other synaptic proteins, possibly SNAP, as suggested by the in vivo FRET analysis presented in a later section.
Figure 3.4 FRAP analysis of dNSF1 mobility. A) Series of images of stimulated wild-type and comatose boutons taken at specific time points illustrated photobleaching and recovery within the ROI. Note the punctate distribution of dNSF1ST17-EGFP prior to photobleaching and hardly any recovery afterwards. B) FRAP within the ROI (as a percentage of maximal recovery) was plotted as a function of time. Each trace represents the mean values of 5 or 6 independent experiments. Dynamic of fluorescence recovery at wild-type synapses was not affected by stimulation (black traces), indicating unchanged protein mobility of dNSF1-EGFP. In contrast, rate of fluorescence recovery at comatose mutant synapses decreased drastically after stimulation (grey traces), indicating much lower mobility of dNSF1ST17-EGFP after stimulation.
3.2.6 Activity-dependent redistribution SNAP

The preceding findings generated great interest in identifying binding partners for dNSF1 at the PAZ. Because t-SNAREs redistribute to the PAZ in an activity-dependent manner in comatose (Kawasaki and Ordway, 2009), and because of the established role for SNAP in promoting binding of NSF to SNAREs (Weidman et al., 1989; Clary et al., 1990), synaptic distribution of Drosophila SNAP was examined at larval neuromuscular synapses.

Our initial immunocytochemical analysis using an anti-dSNAP polyclonal antibody confirmed previous studies demonstrating a predominantly cytosolic distribution of dSNAP within presynaptic boutons (Babcock et al., 2004). To determine whether dSNAP, like dNSF1, exhibits activity-dependent redistribution to the PAZ in comatose, presynaptic expression of EGFP-dSNAP and live imaging were employed to examine the dSNAP distribution before, during and after synaptic stimulation. Notably, 1 minute of 20 Hz stimulation at 33 °C in comatose produced either no redistribution of EGFP-dSNAP or only a modest and transient redistribution which recovered immediately after stimulation (Figure 3.S3). However, increasing the stimulation frequency to 80 Hz produced a punctuate EGFP-dSNAP pattern which resembled that of dNSF1 and persisted long after the stimulation train (Figure 3.5 A lower panel). At wild type synapses maintained at 33 °C, 1 minute of 80 Hz stimulation had little impact on the distribution of EGFP-dSNAP (Figure 3.5 A upper panel). Activity-dependent redistribution of EGFP-dSNAP was quantified as described for dNSF1 (Figure 3.5 B). The respective mean FWHM values for wild type and comatose were 11.1 ± 0.75 (n=6) and 19.1 ± 2.19 (n=6), which were significantly different (p<0.01) (Figure 3.5 C). Finally, PAZ localization of redistributed EGFP-dSNAP was confirmed by immunochemistry. Following 1 minute of 80 Hz stimulation in comatose at 33 °C, puncta of EGFP-dSNAP were localized adjacent to active zones (Figure 3.S4).

Activity-dependent redistribution of the t-SNARE proteins, SYX and SNAP-25, was first observed in comatose at adult DLM neuromuscular synapses and revealed a role for NSF in maintaining active zone t-SNAREs during synaptic activity (Kawasaki and Ordway, 2009). Consistently, redistribution of t-SNAREs to the PAZ was observed at comatose larval neuromuscular synapses after 1 minute of 80 Hz stimulation at 33 °C (Figure 3.S5). As observed for dSNAP, 1 minute of 20 Hz stimulation under the same conditions did not elicit redistribution of t-SNAREs (data not shown). At larval synapses, 1 minute of stimulation at 80 Hz produced significantly more evoked vesicle fusion than at 20 Hz. Therefore, activity-dependent redistribution of t-SNAREs and dSNAP in comatose may reflect their marked accumulation in PAZ complexes at the higher stimulation frequency.
Figure 3.5 Activity-dependent redistribution of SNAP in *comatose*. A) Activity-dependent redistribution of SNAP was examined through live imaging of neurally expressed EGFP-dSNAP in a wild-type or *comtST17* background. In each case, a presynaptic bouton was imaged once at 20 °C (A), and then multiple times at indicated time points (B-E) at 33 °C. 1 minute of 80 Hz stimulation was applied after the preparation had been exposed to 33 °C for 2 minutes. B) Image D (during stimulation) was subtracted from Image C (before stimulation) after correction for photobleaching. Intensity differences for each pixel were plotted in histograms and fit with Gaussian curves. C) The respective full width at half maximum (FWHM) for wild type and *comatose* were $11.1 \pm 0.75$ (n=6) and $19.1 \pm 2.19$ (n=6), which were significantly different from each other (p<0.01).
3.2.7 Redistributed SNAP exhibits reversible association with the PAZ

FRAP studies were carried out to assess whether the mobility of dSNAP changes following its redistribution. EGFP-dSNAP was expressed in a comatose mutant background and FRAP analysis was applied to both resting and stimulated (80 Hz for 1 minute) boutons at 33 °C essentially as described for dNSF1 (see 3.2.5). Exemplary images are shown in Figure 3.6 A. As expected, 80 Hz stimulation elicited redistribution of dSNAP into a stable punctate pattern. However, EGFP-dSNAP puncta exhibited clear recovery after photobleaching (Figure 3.6 B) in contrast to the observed immobilization of dNSF1 under similar conditions. A mild reduction in the mobility of EGFP-dSNAP was observed following stimulation (Figure 3.6 B) which may reflect enhanced binding of dSNAP and dNSF1 under the restrictive conditions in comatose as suggested by FRET studies presented in a later section. The relatively high mobility of dSNAP after its activity-dependent redistribution in comatose indicates that it can bind reversibly at the PAZ and excludes dSNAP as a PAZ anchor for immobilization of dNSF1. Furthermore, the requirement for 80 Hz stimulation to elicit redistribution of dSNAP and SNARE proteins to the PAZ, in contrast to clear dNSF1 redistribution and immobilization elicited at 20 Hz, suggests that dNSF1 binding partners beyond the 20S particle participate in immobilization of dNSF1 at the PAZ.
Figure 3.6 FRAP analysis of SNAP mobility. Mobility of EGFP-dSNAP expressed in a comt<sup>ST17</sup> background was assessed at 33 °C with and without 80 Hz stimulation. A) Images of a stimulated bouton taken at specific time points illustrated photobleaching and recovery within the ROI. Note the punctate distribution of EGFP-dSNAP prior to photobleaching and recovery of the fluorescent puncta afterwards. To emphasize, 2 recovered puncta were circled in the last image. B) FRAP within the ROI (as a percentage of maximal recovery) was plotted as a function of time. Each trace represents the mean values of 7 independent experiments. The percentage fluorescence recovery within 1 minute decreased from 100% before stimulation (black trace) to about 80% after stimulation (grey trace).
3.2.8 *In vivo* interaction between dNSF1 and SNAP revealed by FRET

The preceding findings, together with previous work (Kawasaki and Ordway, 2009), suggest that 20S particles composed of dNSF1, dSNAP and SNAREs are disassembled by dNSF1 activity at the PAZ. Ideally, such inter-protein interactions may be confirmed and characterized within living presynaptic boutons to further define the molecular mechanisms of neurotransmitter release. The current study has achieved this goal for interactions of dNSF1 and dSNAP by combining Fluorescence Resonance Energy Transfer (FRET) approaches (Erickson *et al*., 2001) with transgenic expression of fluorescently tagged synaptic proteins at native synapses. The donor and acceptor fluorophores selected for this study were EGFP and tandem dimer Tomato (tdTomato) (Erickson *et al*., 2003). In designing dNSF1 and dSNAP fusion proteins, consideration was given to biochemical and ultrastructural evidence indicating binding of the SNAP C-terminal and NSF N-terminal domains and their close proximity within the 20S particle (Nagiec *et al*., 1995; Barnard *et al*., 1997; Hanson *et al*., 1997; Hohl *et al*., 1998a; Wimmer *et al*., 2001). Therefore, to maximize FRET, fluorescent proteins were appended to the N-terminus of dNSF1 (EGFP-dNSF1 or the TS mutant form, EGFP-dNSF1ST17) and the C-terminus of dSNAP (dSNAP-tdTomato).

EGFP-dNSF1 or EGFP-dNSF1ST17 was co-expressed with dSNAP-tdTomato in presynaptic boutons of wild-type or *comatose* larvae, respectively. FRET was assessed using the 3-Cube FRET method (Erickson *et al*., 2001). Live imaging of individual presynaptic boutons employed imaging configurations corresponding to donor, acceptor and FRET cubes. These were processed for measurement of a FRET ratio (*FR*), which is a unitless index reflecting the fractional increase in acceptor emission due to FRET (Erickson *et al*., 2001). Notably, a *FR* >1 indicates FRET, whereas a *FR* =1 indicates no FRET (Erickson *et al*., 2001; Erickson *et al*., 2003). First, FRET was measured at resting synapses. An *FR* was calculated for each bouton and a mean *FR* was calculated by averaging multiple boutons from at least 4 preparations. As shown in Figure 3.7 A, clear FRET was detected at the restrictive temperature of 33 °C in *comatose* [*FR*=1.2 ± 0.01 (n=32)]. In contrast, no FRET was detected in *comatose* at 20 °C or wild type at either temperature. The same *FR* of 1.0 ± 0.01 was measured under each of the latter conditions (*comt* 20 °C, n=16; WT 20 °C, n=17; WT 33 °C, n=19). These findings indicate that dNSF1 and dSNAP exhibit increased binding interactions at restrictive temperature in *comatose* in the absence of synaptic stimulation and redistribution of dNSF1 and dSNAP to the PAZ.

Finally, FRET studies were extended to conditions producing activity-dependent redistribution of dNSF1 and dSNAP to examine whether increased interaction was observed as predicted for accumulation of 20S particles at the PAZ. Presynaptic boutons were incubated for 5 minutes at 33 °C with 80 Hz stimulation applied during the second minute, and FRET was measured at precisely 1, 2, 2.5, 3, 4 and 5 minutes (Figure 3.7). Mean *FRs* were determined for individual time points and plotted over time. As shown in Figure 3.7 B, stimulation of wild-type synapses produced no change in FRET as indicated by *FRs* fluctuating around 1.0 (n=6, open circle). In contrast, *comatose* synapses exhibited an activity-dependent increase in FRET during stimulation as indicated by a progressive increase in *FR* from approximately 1.2 to 1.3 during the stimulation train (n=13) (Figure 3.7...
B). In contrast, no significant change in FRET was observed at *comatose* synapses in the absence of synaptic stimulation. Rather, *FRs* fluctuated around 1.2 (n=12,) as expected from FRET studies of resting synapses (Figure 3.7 A). Comparisons of *FRs* at the 2, 2.5, 3, 4 and 5 minute time points with *FRs* at 1 minute (prior to stimulation) indicated that *comatose* exhibited a statistically significant increase in *FR* at the 3, 4 and 5 minute time points (p<0.05).

The above observations demonstrate that acute disruption of dNSF1 activity at *comatose* synapses enhances binding interactions of dNSF1 and dSNAP independent of evoked synaptic activity. Moreover, this interaction is enhanced by intensive synaptic stimulation sufficient to produce redistribution of dNSF1 and dSNAP to the PAZ. Enhanced FRET may reflect the accumulation of more dNSF1 and dSNAP molecules participating in binding interactions. Taken together with parallel FRAP studies, these findings support a model in which dSNAP redistribution to the PAZ involves its reversible binding to dNSF1 and likely other components of 20S particles.
Figure 3.7 In vivo interaction between dNSF1 and dSNAP revealed by 3-Cube FRET measurement. Three-Cube images were taken at individual presynaptic boutons co-expressing dSNAP-tdTomato with EGFP-dNSF1 (in a wild-type background) or EGFP-dNSF1ST17 (in a comatose mutant background). A FR was calculated for each bouton. A) FRET was measured at resting synapses. For wild type, the respective mean FRs were both 1.0 ± 0.01 at 20 °C (n=17) and 33 °C (n=19), whereas for comatose, the respective mean FRs were 1.0 ± 0.01 at 20 °C (n=16) and 1.2 ± 0.01 at 33 °C, which is significantly higher than the other three groups (n=32, p<0.001). B) Activity-dependent increase in FRET was observed at stimulated comatose synapses (black circle) during the 80 Hz stimulation. Each marked with an asterisk, the mean FRs at 3, 4 and 5min were significantly bigger than the initial mean FR at 1min (n=13 p≤0.01). Stimulated wild-type synapses (open circle) and resting comatose synapses (grey circle) exhibited no significant change in FRET associating with the stimulation. The FRs fluctuated around 1.0 (n=6) and 1.2 (n=13) respectively through out the experiments.
3.3 Discussion

Our recent work has suggested a model for the spatial organization of SNARE protein cycling in neurotransmitter release, in which dNSF1 activity mediates SNARE complex disassembly at the PAZ (Kawasaki and Ordway, 2009). The current work builds on this model through live imaging studies exploring the spatial distribution and interactions of dNSF1 and dSNAP within presynaptic boutons and their dependence on synaptic activity.

First of all, co-IP studies demonstrated for the first time that acute disruption of dNSF1 activity in 

comatose
leads to accumulation of protein complexes containing dNSF1, dSNAP and SNAREs. These findings suggest that dNSF1

ST17
is capable of associating in complexes with dSNAP and SNARE proteins but is impaired in its ability to disassemble them. This may reflect the location of the 

comatose

ST17
missense mutation, which changes an amino acid in the D1 domain thought to participate in complex disassembly and may not affect SNAP/SNARE interactions mediated by the N domain (Whiteheart et al., 1994; Nagiec et al., 1995).

Moreover, dNSF1, dSNAP and t-SNAREs exhibit activity-dependent redistribution to PAZ regions of the plasma membrane in 

comatose
. These findings are consistent with previous studies of adult neuromuscular synapses, in which activity-dependent redistribution of t-SNAREs to the PAZ was observed in 

comatose
(Kawasaki and Ordway, 2009). Notably, the distribution of dNSF1 at adult neuromuscular synapses is largely restricted to PAZ regions of the plasma membrane under normal conditions in wild type (not shown). Thus redistribution of dNSF1 to the PAZ cannot be observed in this preparation. Nevertheless, these observations further implicate the PAZ as the location where NSF-mediated disassembly of SNARE complexes would normally take place and where 20S fusion complexes would accumulate when NSF function is disrupted.

As suggested by FRET studies, redistribution of dSNAP may be mediated by enhanced binding with dNSF1. Persistent redistribution of dSNAP and both t-SNAREs occurs following intensive stimulation at 80 Hz. The fact that dSNAP exhibits a stable punctate distribution under these conditions but is still mobile and can disassociate from the PAZ suggests dSNAP may bind and disassociate from dNSF1 and SNAREs independent of SNARE complex disassembly. Moreover, the apparent shift of this equilibrium toward dSNAP binding to the PAZ may reflect accumulation of dNSF1 and t-SNAREs, which are thought to bind dSNAP separately or together in 20S complexes, leading to higher capacity of affinity for dSNAP binding at the PAZ.

Unlike dSNAP, dNSF1 is fixed at the PAZ following its redistribution in 

comatose
. This observation suggests that dNSF1 may be anchored or immobilized at the PAZ by synaptic components which themselves are immobile. For this reason and because dNSF1 may be immobilized at the PAZ in the apparent absence of dSNAP or SNARE redistribution, synaptic components other than SNAP and SNAREs appear to anchor dNSF1 at the PAZ.

Taken together, the results reported here further define the in vivo behavior and interactions
of NSF, SNAP and SNARE proteins at the core of the neurotransmitter release apparatus. These studies benefited from a powerful combination of genetic tools including a TS NSF mutant, comatose, and transgenic lines permitting cell-type specific expression of fluorescent fusion proteins at native synapses. Live imaging studies revealed activity-dependent redistribution of dNSF1 and dSNAP proteins to the PAZ following disruption of dNSF1 function and characterized their protein mobilities there. For the first time in any system, intermolecular FRET was observed between presynaptic proteins and defined activity-dependent protein interactions within living presynaptic boutons. The resulting findings further support and extend a model in which the spatial organization of SNARE protein cycling in neurotransmitter release includes NSF-dependent disassembly of SNARE complexes at PAZ regions of the presynaptic plasma membrane.

3.4 Supplementary figures

Figure 3.S1 Loss of dNSF1 staining in comt^{CLP1}. Specificity of anti-dNSF was examined with immunofluorescence imaging analysis. Preparation of the dNSF1 null mutant, comt^{CLP1}, was labeled with anti-HRP, anti-dNSF and anti-CSP. Note the loss of dNSF1 staining in panel b, which indicated anti-dNSF is specific for dNSF1.
Figure 3.S2 Lack of redistribution of dNSF1\textsuperscript{ST17}-EGFP under the permissive condition of \textit{comatose} mutation. Presynaptic boutons expressing dNSF1\textsuperscript{ST17}-EGFP in a comt\textsuperscript{ST17} background were subjected to live-imaging analysis at the permissive temperature of 20 °C. A bouton was imaged at indicated time points (A-E). 1 minute of 20 Hz stimulation was applied from 2 to 3 minute. Note the lack of redistribution of dNSF1\textsuperscript{ST17}-EGFP upon stimulation.

Figure 3.S3 Transient redistribution of EGFP-dSNAP upon 20 Hz stimulation. Presynaptic boutons expressing EGFP-dSNAP in a comt\textsuperscript{ST17} background were subjected to live-imaging analysis at the restrictive temperature of 33 °C. A bouton was imaged once at 20 °C (A), and then multiple times at 33 °C at indicated time point (B-E). 1 minute of 20 Hz stimulation was applied after the preparation had been exposed to 33 °C for 2 minutes. Note the transient redistribution EGFP-dSNAP upon stimulation and the distribution recovery afterwards.
Figure 3.S4 PAZ localization of EGFP-dSNAP after activity-dependent redistribution in *comatose*. Presynaptic terminals expressing EGFP-dSNAP in a *com*<sup>ST17</sup> background were subjected to confocal immunofluorescence imaging analysis. Synapses were kept at rest (a, b) or subjected to 1 minute of 80 Hz stimulation (c, d) at 33 °C prior to fixation, and distribution of EGFP-dSNAP (green) at was determined respect to presynaptic active zones (red, stained with anti-BRP antibody). Note the punctate distribution pattern and PAZ localization of EGFP-dSNAP after stimulation. All images represent maximum projections of 2 optical sections.
Figure 3.S5 PAZ localization of t-SNAREs upon 80 Hz stimulation in comatose. Distributions of endogenous SNAP-25 and SYX were examined with confocal immunofluorescence imaging. ComfST17 boutons were subjected to 1 minute of 80 Hz stimulation at 33 °C prior to fixation. The presynaptic plasma membrane was labeled with anti-HRP (a, f), presynaptic active zones were visualized with anti-BRP (c) or CAC-tdTomato (h) and endogenous SNAP-25 (b) and SYX (g) were labeled with specific antibodies respectively. Note the punctate distribution patterns and PAZ localizations of SNAP-25 and SYX. All images represent maximum projections of 2 optical sections.
Chapter 4  *In vivo* Fluorescence Resonance Energy Transfer (FRET) analysis of synaptic protein interactions during neurotransmitter release

4.1 Introduction

FRET refers to the Förster Resonance Energy Transfer. Named after the German scientist Theodor Förster, it describes the phenomenon of non-radiative form of energy transfer between two chromophores. When both chromophores are fluorescent molecules (fluorophores), like in this dissertation, the term “Fluorescence Resonance Energy Transfer” is used instead.

Briefly, a fluorophore in its electronic excited state may transfer its energy to be accepted by a second fluorophore in close proximity. While the first fluorophore is known as the donor, the second fluorophore is known as the acceptor. FRET is always unidirectional, that is from the donor to the acceptor fluorophore.

Efficiency of the energy transfer, also known as the FRET efficiency ($E$), is defined as the quantum yield of the energy transfer transition, which equals the fraction of energy transfer events occurring per donor excitation event (Förster, 1948). Importantly, as far as this project concerns, FRET efficiency mainly depends on 2 factors:
1. The overlap of the donor emission spectrum and the acceptor excitation spectrum.
2. The distance between donor and acceptor fluorophores.

To understand the importance of spectral overlap for FRET, consider the energy states that an electron of a fluorescent molecule goes through during excitation and emission. During excitation, light energy (in the form of photons) within a certain range of wavelengths (represented by the excitation spectrum of the fluorophore) is absorbed to excite electrons of the fluorescent molecule into an orbital that is farther away from the nucleus. This represents a higher energy state of the electron. An electron that has transited to a higher energy state is unstable and has the tendency to release energy and return to the ground state. One return pathway is through fluorescing, that is to release photons of longer wavelengths (represented by the emission spectrum of the fluorophore). Alternatively, the fluorophore may transfer energy to another fluorophore through FRET. Because spectral property determines that a fluorescent molecule only absorbs or emits energy of same quantal sizes as photons of certain wavelengths, FRET may occur only if the emission spectrum of the donor fluorophore overlaps substantially with the excitation spectrum of an acceptor fluorophore present in close proximity. Under this condition, instead of photon emission, electron energy may be transferred non-radiatively to the acceptor through FRET.

With regard to distance, FRET is a very short range effect that occurs only on the nanometer scale. The energy transfer efficiency decreases as the two fluorophores separate further (Förster, 1948). The experimentally measured Förster distance of a FRET pair (the distance between the donor and acceptor when 50% FRET efficiency is reached) is typically around 30 Å (Stryer and Haugland, 1967). FRET is usually non-existent at an intermolecular distance beyond 10 nanometers. Therefore, detecting FRET is a trusted indicator of molecular range proximity of the donor and acceptor, and strongly suggests actual physical contact between...
the two. According to Stryer and Haugland, FRET can serve as a good optical ruler with molecular resolution within the range of 10-60 Å (Stryer and Haugland, 1967). In the field of biomedical sciences, FRET has become a common measure for detecting and quantifying direct binding interactions between macromolecules (such as DNA and proteins) with suitably matching FRET pairs attached. The application of genetically encoded fluorophores (Shaner et al., 2004; Shaner et al., 2005) has fostered even greater progress in the in vivo study of protein-protein interaction. Intracellular expression of genetically engineered fusion proteins, comprising of such fluorophores and proteins of interest, can be achieved in various experimental settings without much interference with normal protein function or other cellular activities.

Commonly used methods of FRET measurement can be categorized as fluorescence intensity-based and fluorescence lifetime-based methods (Erickson et al., 2001; Parsons et al., 2004; Zal and Gascoigne, 2004; Voss et al., 2005; Greeson et al., 2006). A third type of FRET measurement based on the donor photobleaching rate is also discussed below (Jovin and Arndt-Jovin, 1989). FRET can be quantified experimentally to give the FRET efficiency ($E$), which is indicative of the donor-acceptor distance and thus the molecular interaction.

As mentioned above, FRET is a nonradiative process, that is, the energy transfer process does not involve the emission and re-absorption of photons. Therefore, FRET reduces the quantum yield of the donor, which is the ratio of fluorescence emission to nonradiative energy loss (Lichtman and Conchello, 2005). Such changes in donor quantum yield or the total light emission over the entire fluorescent spectral range can be quantified experimentally by measuring the fluorescence intensity the donor before and after the acceptor molecule is photobleached to block FRET.

The FRET efficiency relates to the quantum yield as follows:

$$E = 1 - \frac{F_{DA}}{F_D}$$

where $E$ is the FRET efficiency, $F_{DA}$ and $F_D$ are donor fluorescence intensity in the presence and absence of an acceptor, respectively.

Acceptor photobleaching FRET, or apFRET, is one of two intensity-based FRET methods involved in our studies. Another widely used intensity-based measurement is sensitized emission FRET, or seFRET, which measures the acceptor fluorescence emission change due to FRET. In the absence of FRET, donor emission is observed upon donor excitation. The acceptor emission is almost zero during donor excitation because of the spectral shift between the two fluorophores (Figure 4.1 A). In the presence of FRET, the acceptor fluorophore gets excited by the energy absorbed from the donor and emits fluorescence of longer wavelengths, and acceptor emission may be detected upon donor excitation. This provides a convenient FRET measurement through monitoring the acceptor emission change in the presence or absence of the donor.
After years of development, intensity-based FRET measurements have been successfully adapted to various experimental systems with corresponding mathematic algorithms for data processing and error correction (Erickson et al., 2001; Erickson et al., 2003; Zal and Gascoigne, 2004; Wallrabe and Periasamy, 2005; Greeson et al., 2006). In addition, these relatively easy and economical measurements for FRET are performable on most prevalent lab fluorescence imaging systems, and have been commonly used in biomedical research.

Intensity-based methods have several pitfalls. For example, one inconvenience of the acceptor photobleaching FRET method is that photobleaching permanently fades the fluorescence signal of the acceptor, and increases the difficulty of performing repeat tests. Also, heat and free-radicals produced by repeated high-power laser scanning during photobleaching cause damage to the tissue structure and intracellular environment, which is especially unwanted in studies of living organisms or cells. On the other hand, while the non-destructive sensitized emission FRET method is often the preferred intensity-based method, it is limited by the properties of optical filters and suffers from signal contamination caused by spectral bleed-through or channel cross-talk. However, methods have been developed to eliminate the cross-talk effects by incorporating several coefficients that represent ratios of emission through different channels when one fluorophore is present without the other (Youvan et al., 1997; Gordon et al., 1998; Erickson et al., 2001; Xia and Liu, 2001). These algorithms of FRET quantification are far more complicated and involve greater amount of work.

Sensitivity of detection for intensity-based FRET methods is greatly affected by fluorophore concentrations. First of all, the fluorescent signals of both fluorophore species need to be robust enough to minimize the influence of noise. Moreover, concentrations of both fluorophore species need to be determined or controlled at a constant level, because these methods cannot distinguish elevated FRET caused by an increase in fluorophore numbers (fluorophore concentration) from that due to increased binding (binding efficiency). In addition, given that the fluorophore concentrations and the binding efficiency of the pair are both constant, sensitivity of FRET detection could also be affected by the donor-to-acceptor ratio. Specifically, apFRET measurement should have higher detection sensitivity when the acceptor molecules are present in excess of the donor. Ideally, when the entire donor population is occupied by acceptors (donor saturation), photobleaching of acceptors causes every donor molecule to be dequenched and fluoresce more to produce the most robust FRET signal. On the contrary, a molecular ratio approaching acceptor saturation should be more suitable for seFRET measurement, because, in such circumstances, emission from almost every acceptor molecule is increased (sensitized) due to FRET. This would result in the most robust FRET signal by this method.

In addition to intensity-based methods, other techniques for FRET measurement include the fluorescence lifetime imaging microscopy (FLIM). Fluorescence lifetime is the average time (in nanosecond scale) a molecule stays excited before yielding a photon and decaying to the ground state (Lichtman and Conchello, 2005). It is an intrinsic property characteristic to the individual fluorophore. Fluorescence lifetime is influenced by the intracellular environment
in the immediate vicinity of the molecule, such as changes in ion concentrations, the environmental PH and FRET.

The FRET efficiency relates to the fluorescence lifetime of the donor molecule as follows:

\[ E = 1 - \frac{T_{DA}}{T_D} \]

where \( E \) is the FRET efficiency, \( T_{DA} \) and \( T_D \) are donor fluorescence lifetime in the presence and absence of an acceptor, respectively.

There are two main methods for measuring the fluorescence lifetime with FLIM, namely, time- and frequency-domain FLIM (Elson et al., 2004; Suhling et al., 2005). The former technology excites samples using a pulsed light source with pulse duration of a few hundred picoseconds and the resultant emission is captured with a nanosecond-level shutter to calculate the emission decay rate that is then converted into the excitation lifetime. The frequency-domain FLIM excites samples using modulated light of megahertz frequencies, and because the excitation state has a lifetime, the emission will be delayed with respect to the excitation. Fluorescence lifetime can then be calculated based on this phase shift (Wallrabe and Periasamy, 2005).

Compared to fluorescence intensity-based measurements, FLIM is a relatively challenging technology. However, it is a highly sensitive measurement of FRET. It probes the change in the donor excitation lifetime or the rate of decay of emission and is therefore not affected by the concentration of FRET species, excitation intensity or other factors that limit the intensity-based measurements discussed above. Moreover, because FLIM is independent of the spectral emissions of the FRET species, it is capable of imaging fluorophores of overlapping emissions simultaneously. This greatly broadens the application and combination of fluorophores in FRET studies.

Finally, another time-based measurement of FRET is based on the photobleaching rate of the donor fluorophore. Resonance energy transfer from an excited donor to an acceptor fluorophore prevents the photobleaching of that donor fluorophore (Jovin and Arndt-Jovin, 1989), and therefore robust FRET leads to a longer photobleaching decay time. Experimental determination of the FRET efficiency with this method is relatively simple and can be performed on most fluorescence microscopes. Briefly, samples are excited with the donor excitation wavelength (with minimal excitation of the acceptor) and the fluorescence emission of the donor in the presence and absence of an acceptor is monitored overtime. And the FRET efficiency is given by:

\[ E = 1 - \frac{T_{pb}}{T_{pb}'} \]

where \( E \) is the FRET efficiency, \( T_{pb}' \) and \( T_{pb} \) are the photobleaching decay time constant of the donor in the presence and absence of an acceptor, respectively. Note that the fraction in this equation is the reciprocal of that used for the lifetime measurement.
One advantage of the photobleaching time measurement is that the donor photobleaching time duration is at a scale of second or minute rather than nanosecond, making it easier than the fluorescence lifetime measurement. Similar to the fluorescence lifetime, photobleaching decay rates do not depend on fluorophore concentrations, therefore careful control for concentrations of the FRET species is not needed. However, because of the spectral bleed-through issue, the optical filter setting has to be designed carefully. Also, illumination has to be kept constant throughout the measurement, because the photobleaching rate of the individual fluorophore changes markedly with the intensity of excitation.

4.2 Objectives of the current FRET study.

Discovery, improvements and application of genetically encoded fluorescent proteins have provided an exciting opportunity to study in vivo protein interaction with FRET, because a fluorescent protein can be fused to an end of a protein of interest through genetic engineering and expressed in vivo as a fluorescent tag of the fusion peptide. A major objective of our lab has been to develop in vivo FRET analysis in the Drosophila model system to analyze neuronal protein interactions at native synapses. Clear background of Drosophila genetics and convenient tools for in vivo gene and protein manipulation in this model system have greatly facilitated the application of in vivo FRET.

The current FRET study has been focused on analyzing interactions among SNAREs, NSF and SNAP. This is because active interactions among these proteins during synaptic transmission, including their assembly into a 20S fusion particle, have been supported by abundant biochemical data. Therefore, there is a good chance of detecting in vivo FRET produced by these pairs of proteins. In addition, ultrastructural studies have revealed the organization of subunits in the 20S particle (Figure 1.8). According to their relative orientation in assembly with each other, fluorescent protein tags can be selectively fused to the N- or C- termini of proteins of interest to optimize the robustness of FRET signals.

The 20S fusion particle is thought to be at the core of SNARE-mediated synaptic vesicle fusion and neurotransmitter release. However, the proposed model of its assembly, organization, and NSF-dependent disassociation is based mostly on in vitro biochemical and ultrastructural analysis. A major objective of the current study is to visualize these processes as they take place at living synapses and thus to produce in vivo evidence supporting or challenging current models. The main focus of the entire dissertation project has been on the biological significance of NSF-dependent disassociations of cis-SNARE complex and the 20S fusion particle. The current study has utilized acute inactivation of NSF in the temperature sensitive comatose mutant, and applied FRET analysis to different combinations of candidate binding partners including SNAREs, NSF and SNAP.

4.3 Methodology for in vivo FRET analysis in Drosophila

4.3.1 Selection of fluorophores to serve as the FRET pair

A prerequisite condition for the success of the FRET project is that the selected pair of FRET
donor and acceptor fluorophores must be suitable for the experimental system. Due to the nature of this study, this important preliminary work involves choosing from various fluorescent protein species based on the molecule size, brightness, photostability and spectral properties (Shaner et al., 2005), cloning the gene, generating transgenic fly lines that express the fluorescent protein at a suitable level when driven by the selected GAL4 driver, and test imaging the fluorophore in a dissected animal under the available fluorescence imaging system. An ideal fluorophore should be bright enough when imaged at individual synaptic terminals and have a good photostability to prevent photobleaching at a fast rate; expression and maturation of the fluorophore must not be toxic to cells and does not interfere with the development of the organism. To produce robust FRET, there must be enough overlapping of the donor emission and acceptor excitation spectrum; however, to maximally avoid channel bleed-through, a good spectral separation of the donor and acceptor is also desired.

In the beginning, the enhanced green fluorescent protein (EGFP) and the Discosoma sp. red fluorescent protein (DsRed) were selected as the donor and acceptor fluorophores respectively based on the above criteria. A comparison of related properties of commonly used fluorescent protein variants is provided in Table 4.1. However, DsRed has been proven to be an obligate tetramer in vitro (Baird et al., 2000) and has a strong tendency to form large-scale molecule aggregations when expressed in cells (Jakobs et al., 2000; Lauf et al., 2001; Mizuno et al., 2001). Similar artifacts were observed in this project. For example, several DsRed fusion proteins expressed in vivo exhibited widespread punctate concentrations under microscopes, suggesting severe interference to cellular localizations of those target proteins.

Because of this artifact, the tandem dimer tomato (tdTomato) was selected as the acceptor fluorophore instead. TdTomato is a tandem dimer molecule of the genetically engineered DsRed variant, Tomato (Shaner et al., 2004). Intracellular dimerization of tdTomato eliminates the tendency for further oligomerization or aggregation of this molecule (Campbell et al., 2002; Shaner et al., 2004). The spectral distribution of tdTomato is very similar to that of DsRed, with peak excitation and emission of 554 nm and 581 nm respectively (Shaner et al., 2004). Moreover, tdTomato has greatly improved brightness and photostability (Table 4.1) as well as a 10-time faster maturation speed than DsRed (Shaner et al., 2004), making it an ideal FRET acceptor for EGFP. One drawback is that the much improved brightness of tdTomato is at the expense of doubling the molecular weight. However, cellular toxicity or interference to the target protein localization or structure caused by tdTomato tags hasn’t been observed in this project yet.
Table 4.1 Properties of the best fluorescent protein variants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Excitation(^a) (nm)</th>
<th>Emission(^b) (nm)</th>
<th>Brightness(^c)</th>
<th>Photostability(^d)</th>
<th>Oligomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>587</td>
<td>610</td>
<td>16</td>
<td>96</td>
<td>Monomer</td>
</tr>
<tr>
<td>tdTomato</td>
<td>554</td>
<td>581</td>
<td>95</td>
<td>98</td>
<td>Tandem dimer</td>
</tr>
<tr>
<td>mStrawberry</td>
<td>574</td>
<td>596</td>
<td>26</td>
<td>15</td>
<td>Monomer</td>
</tr>
<tr>
<td>DsRed-monomer</td>
<td>556</td>
<td>586</td>
<td>3.5</td>
<td>16</td>
<td>Monomer</td>
</tr>
<tr>
<td>Venus</td>
<td>515</td>
<td>528</td>
<td>53</td>
<td>15</td>
<td>Weak dimer</td>
</tr>
<tr>
<td>EYFP</td>
<td>514</td>
<td>527</td>
<td>51</td>
<td>60</td>
<td>Weak dimer</td>
</tr>
<tr>
<td>EGFP</td>
<td>488</td>
<td>507</td>
<td>34</td>
<td>174</td>
<td>Weak dimer</td>
</tr>
<tr>
<td>ECFP</td>
<td>433</td>
<td>475</td>
<td>13</td>
<td>64</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

\(^a\)Major excitation peak. \(^b\)Major emission peak. \(^c\)Product of excitation coefficient and quantum yield at pH 7.4. \(^d\)Time for photobleaching from an initial emission rate of 1,000 photos/s down to 500 photos/s (t\(_{1/2}\)). Modified from Shaner NC \textit{et al}. (2005).

4.3.2 Generation of transgenic lines for \textit{in vivo} FRET analysis

4.3.2.1 Generation of recombinant chromosomes containing a transgene insertion and a loss-of-function mutation

In this study, presence of endogenous proteins (without fluorescent fusion tags) in great amount could reduce the sensitivity of FRET detection, because they are likely to compete with exogenous fusion proteins for an \textit{in vivo} binding partner. Moreover, such undesired binding may even have a higher efficiency than binding of fusion proteins. To avoid this problem, a chromosome with a transgenic insertion (transgene) is sometimes recombined with a loss-of-function mutant allele of the correspondent gene to reduce the amount of endogenous protein present in synaptic terminals. Recombinant chromosomes generated for this purpose are summarized in Table 4.2

Table 4.2 Recombinant chromosomes containing a transgenic insertion and a loss-of-function mutant.

<table>
<thead>
<tr>
<th>Endogenous gene</th>
<th>Chromosome Number</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYX</td>
<td>3rd</td>
<td>tdTomato-SYX (196A) SYX(^{A229})</td>
</tr>
<tr>
<td>NSYB</td>
<td>3rd</td>
<td>EGFP-NSYB(28A) NSYB(^{AF33B}) tdTomato-NSYB(28A) NSYB(^{AF33B})</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>3rd</td>
<td>EGFP-SNAP25(107B) SNAP-25(^{MX124}) tdTomato-SNAP25(236C) SNAP-25(^{MX124})</td>
</tr>
</tbody>
</table>

\* Genotypes include names of transgenic insertion lines in parentheses (named by the Ordway Lab).

4.3.2.2 Selection of a GAL4 driver for neuronal expression of fluorescent fusion proteins

To achieve pan-neural expression of the fluorescent fusion proteins engineered for this
project, several X-lined neuronal GAL4 drivers were tested to drive expressions of fluorescent protein genes engineered downstream of the UAS sequence. Among them, the enhancer-trap line of elav-Gal4 driver produces high levels of expressions at all developmental stages (Robinow and White, 1988). A second driver, Appl-Gal4 was also found to produce robust expressions at both larval and adult stages with especially stronger expression in adults (Torroja et al., 1998). Both drivers were used to drive transgenic expression in this project. Also, recombinant X-chromosomes containing both drivers were generated to achieve overall high expression throughout development.

4.3.2.3 Crosses to generate transgenic flies co-expressing the FRET donor and acceptor in the nervous system

Fly lines carrying 2 types of transgenes, whose products are the FRET donor and acceptor respectively, were crossed to GAL4 driver lines to produce progeny that co-express both proteins in the nervous system. Transgenic expression was driven by the X-linked pan-neural driver, Appl-GAL4. For analysis in a comatose mutant background, Appl-GAL4 was recombined with the X-linked comtST17 allele. Before crossing to GAL4 lines, in some cases a transgenic insertion (transgene) was recombined with a loss-of-function mutant allele of the corresponding gene to reduce the amount of endogenous protein (Table 4.2). The GAL4 driver is X-linked and transgenic insertions needed to be on the same chromosome as endogenous genes, which is usually an autosome, for recombination to occur. Below are 2 exemplary crossing schemes for generating such progeny for FRET measurement. Specifically, Cross 1 has both transgenes (transgene 1 and 2) on the 3rd chromosome, while Cross 2 has one on each of the autosomes. Because transgenes carry a \( w^+ \) marker, all crosses are done in a \( w \) (white) background.
Cross 1:

\[
\begin{array}{ccc}
\text{w} & \text{TM3} & \text{w} \\
\text{TM6} & \text{y} & \text{TM6} \\
\text{w} & \text{FM7} & \text{w} \\
\text{FM3} & \text{y} & \text{FM3} \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{TM3} & \text{transgene} \\
& & 1 \\
\text{w} & \text{FM7} & \text{w} \\
\text{FM3} & \text{y} & \text{FM3} \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{TM3} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{TM3} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{TM3} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{TM3} & \text{transgene} \\
& & 1 \\
\end{array}
\]

Cross 2:

\[
\begin{array}{ccc}
\text{w} & \text{Sfo} & \text{w} \\
\text{w} & \text{Cy} & \text{Yas} \\
\text{FM3} & \text{Cy} & \text{FM3} \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{Cy} & \text{transgene} \\
& & 1 \\
\text{w} & \text{FM3} & \text{Yas} \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{Yas} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{Yas} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{Yas} & \text{transgene} \\
& & 1 \\
\text{w} & \text{Appl-} & \text{YAS-} \\
\text{CAL4} & \text{Yas} & \text{transgene} \\
& & 1 \\
\end{array}
\]
4.3.3 FRET analysis based on the 3-cube FRET measurement

4.3.3.1 The 3-cube FRET algorithm

First developed by David Yue and colleagues, the 3-Cube FRET measurement is an intensity-based assay that quantifies FRET by measuring sensitized acceptor emission. (Erickson et al., 2001).

Specifically, FRET is measured in the FRET ratio \( FR \) in this method, which is given by:

\[
FR = \frac{F_{AD}}{F_A}
\]

where \( F_{AD} \) and \( F_A \) are acceptor fluorescence intensity upon donor excitation with and without a donor respectively. Therefore, when no FRET, \( FR \) should equal to 1 while in the presence of FRET, \( FR \) should be greater than 1.

As discussed earlier, due to spectral bleed-through or channel cross talk, seFRET methods are usually complicated by the inability to selectively excite the donor or selectively detect emission from the acceptor. Consider the FRET pair of CFP and DsRed. Upon excitation of CFP at 488 nm (the spectral peak for CFP excitation), emission detected at 580 nm (spectral peak of DsRed emission) consists of multiple components: DsRed emission due to FRET, DsRed emission due to direct excitation and CFP emission due to direct excitation (Figure 4.1 B). To distinguish among these components in 3-Cube FRET, samples are imaged through filter cubes composed of excitation, dichroic mirror and emission filters that allow (1) optimal excitation and detection of the donor (the donor cube); (2) optimal excitation and detection of the acceptor (the acceptor cube); (3) optimal excitation of the donor and detection of the acceptor (the FRET cube). As described by Erickson et al (Erickson et al., 2001), the designation \( SCUBE(SPECIMEN) \), where CUBE denotes the specific filter cube, and SPECIMEN points out whether the cell is expressing the donor only (\( D \)), acceptor only (\( A \)), or both (\( DA \)), will be used to indicate the imaging configuration.
Figure 4.1 A) Spectral properties favoring CFP and DsRed as FRET partners. Excitation (thick lines) and emission (thin lines) were shown for CFP (black) and DsRed (grey), respectively. B) Emission of CFP and DsRed FRET pair upon 488 nm excitation. Emission at 480 nm has a single component: ⑤, CFP emission at 480 nm. Emission at 580 nm has multiple components: ④, CFP emission at 580 nm, ③, DsRed emission due to direct excitation of 488 nm and DsRed emission due to FRET. ①, Total emission of CFP and DsRed at 580 nm (equals ②+④). ②, Total DsRed emission at 580 nm (equals ③ + DsRed emission due to FRET). Adapted from Erickson et al. (2003).

Excitation at different wavelengths changes the size but not the shape of emission spectrum of a fluorophore. Therefore, for a particular fluorophore under certain imaging configuration, the ratio of fluorescence emission intensity through different filter cubes is a constant value. 3 constant values, \( R_A \), \( R_{D1} \) and \( R_{D2} \) are incorporated in the 3-Cube FRET measurement to eliminate the contribution of signal bleed-through to the final \( FR \). Specifically, when the donor or acceptor is expressed alone in the cell, the constant values can be experimentally determined by the following equations:

\[
R_A = \frac{S_{\text{FRET}}(A)}{S_{\text{ACCEPTOR}}(A)} \quad \text{……………………………………………….}(1)
\]

\[
R_{D1} = \frac{S_{\text{FRET}}(D)}{S_{\text{DONOR}}(D)} \quad \text{……………………………………………….}(2)
\]

\[
R_{D2} = \frac{S_{\text{ACCEPTOR}}(D)}{S_{\text{DONOR}}(D)} \quad \text{……………………………………………….}(3)
\]

After calibrating signal bleed-through with these constant values, the \( FR \) is finally given by:

\[
FR = \frac{F_{AD}}{F_A} = \frac{(S_{\text{FRET}}(AD) - S_{\text{DONOR}}(AD) \times R_{D1})}{[(S_{\text{ACCEPTOR}}(AD) - S_{\text{DONOR}}(AD) \times R_{D2}) \times R_A]} \quad \text{…………..}(4)
\]

In this project, the FRET donor and acceptor are EGFP and tdTomato respectively. Therefore, the above Equation 1-4 can be written as Equation 4-8 respectively:

\[
R_A = \frac{S_{\text{FRET}}(A)}{S_{\text{tdTomato}}(A)} \quad \text{……………………………………………….}(5)
\]

\[
R_{D1} = \frac{S_{\text{FRET}}(D)}{S_{\text{EGFP}}(D)} \quad \text{……………………………………………….}(6)
\]
\[ R_{D2} = S_{\text{tdTomato}}(D)/S_{\text{EGFP}}(D) \] .................................(7)

\[ FR = F_{AD}/F_A = (S_{\text{FRET}}(AD) - S_{\text{EGFP}}(AD) \times R_{D1})/[(S_{\text{tdTomato}}(AD) - S_{\text{EGFP}}(AD) \times R_{D2}) \times R_A] \] ..........................(8)

### 4.3.3.2 Image acquisition, processing and data analysis

In this project, the 3-Cube FRET measurement was combined with confocal fluorescent live-imaging. Microdissection, live-preparation of experimental animals and confocal live-imaging were performed as described in Chapter 2. Fluorescent images were obtained with a Zeiss LSM confocal microscope equipped with a Plan-Apochromat 63x water immersion objective (Zeiss, Germany), an Ar 488 nm laser and a He-Ne 543 nm laser. For dual-color imaging, a Z488/543RPC dichroic mirror was used with HQ520/40 (green) and HQ610/75 (red) emission filters. To generate 3-cube images, samples were excited once with 488 nm laser and then with 543 nm laser, emission through the 2 filters was recorded by separate photomultiplier tubes (PMT). As a result, the 3 cube images are (cube, excitation, emission), EGFP, 488 nm, HQ520/40; tdTomato: 543 nm, HQ610/75 and FRET, 488 nm, HQ610/75, respectively.

In this study, flies of 3 genotypes were used to measure FRET for each donor-acceptor pair. Specifically, flies expressing only the donor fluorescent protein were imaged to measure constant value \( R_{D1} \) and \( R_{D2} \), while those expressing only the acceptor were imaged to measure \( R_A \). In addition, flies co-expressing both fluorescent proteins were imaged to measure FRET (Table 4.3).

Constant value and FRET measurements were performed at individual presynaptic boutons. Typically, a \( FR \) was determined for at least 4 boutons from each preparation and a mean \( FR \) was finally produced by averaging measurements from a minimum of 4 preparations. Specifically, 3-cube images were taken from a 12×12μm square region containing a single bouton (larvae, ~5 μm in diameter) or a few boutons (adult, ~1 μm in diameter). The EGFP cube image was processed first: A region of interest (ROI) was drawn closely to the edge of a bouton. This ROI was then duplicated and place about 3μm away (horizontally) to measure the background fluorescence. Background was then subtracted from the whole bouton fluorescence to produce the final measurement of fluorescence intensity from the EGFP cube, \( S_{\text{EGFP}} \). The tdTomato and FRET cube images were then processed using the same ROIs to produce \( S_{\text{tdTomato}} \) and \( S_{\text{FRET}} \). These measurements were brought into Equation 4-8 to calculate \( R_A \), \( R_{D1} \), \( R_{D2} \) and \( FR \) respectively.

### 4.3.3.3 Error control and correction

In this study, constant values were measured individually for each FRET pair. This is because: 1) the expression level of a transgene driven by GAL4 varies a lot with the location of transgenic insertion. Therefore, one transgenic line was selected for each fluorescent fusion protein of the FRET pair (see list of transgenic lines in Table 4.3), and imaging configurations, such as laser intensity, confocal pinhole size and PMT value, were optimized accordingly for simultaneously imaging the selected donor and acceptor lines. Such configurations, once decided, were then kept exactly the same for constant value and FRET measurements of the particular FRET pair; 2) Cellular localization could possibly affect constant values measured
for a fluorescence fusion protein (Erickson et al., 2001). However, such variation, if present, was too small to be detected in this study. For example, $R_A$ values measured for soluble tdTomato-dSNAP and membrane-anchored tdTomato-SNAP-25 were not significantly different under the same imaging configuration.

Besides the above, a slight shift in the alignment of imaging devices along light path would significantly change results of constant value measurements under certain imaging configuration. Therefore, the imaging system was calibrated as often as needed. Also, FRET ratios were always calculated using constant values taken within the same 24 hours to avoid errors caused by gradual shift in device alignment.

4.3.3.4 Preliminary control experiments

Theoretically, EGFP and tdTomato should make a matching pair of FRET donor and acceptor. However, preliminary studies in our laboratory were required to establish the suitability of this FRET pair for analysis of protein interactions at native Drosophila synapses. Therefore, a concatemer molecule was generated, as shown in Figure 4.2 A, where EGFP and tdTomato are separated by a linker of 20 amino acids. Robust FRET was detected at Drosophila presynaptic terminals expressing this fusion protein. On the contrary, when separate EGFP and tdTomato molecules were co-expressed at the same presynaptic terminal of Drosophila, no FRET was detected (Figure 4.2 B). In this set of control experiments, an acceptor sensitized emission method, 3-cube FRET, was used, where FRET was quantified in FRET ratio ($FR$), the ratio of emission from the acceptor fluorophore with and without a donor. Therefore, a $FR$ of 1 indicates no FRET, while a $FR$ greater than 1 indicates positive detection of FRET. Detailed discussion of the 3-cube FRET measurement will be in later sections of this chapter.

**Figure 4.2 Preliminary control experiments.** A) Illustration of the EGFP-tdTomato concatemer molecule. B) 3-Cube FRET measurement at Drosophila 3rd instar larval neuromuscular synapses. Using the 3-Cube FRET measurement, robust FRET was detected at presynaptic boutons expressing the EGFP-tdTomato concatemer (upper). No FRET was detected from presynaptic boutons co-expressing soluble EGFP and tdTomato fluorophores (lower).

FRET pairs examined by the 3-Cube FRET method and genotypes of experimental animals were provided in Table 4.3.
Table 4.3 Possible synaptic proteins interactions examined through the 3-Cube FRET measurement.

<table>
<thead>
<tr>
<th>Binding Partners</th>
<th>FRET Pair</th>
<th>Dev. Stage</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF and SNAP</td>
<td>NSF-EGFP, tdTomato-SNAP</td>
<td>Larva</td>
<td>w Appl-GAL4/y;;NSF-EGFP(188A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;tdT-SNAP(8A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;NSF-EGFP(188A)/tdT-SNAP(8A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td>w Appl-GAL4/y;;NSF-EGFP(188A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;tdT-SNAP(62B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;NSF-EGFP(188A)/tdT-SNAP(62B)</td>
</tr>
<tr>
<td>NSF and SNAP</td>
<td>EGFP-NSF17, SNAP-tdTomato</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;NSFST17-EGFP(214C)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;tdT-SNAP(8A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;NSFST17-EGFP(214C)/+;tdT-SNAP(8A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td>w Appl-GAL4 comtST17/y;NSFST17-EGFP(214C)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;tdT-SNAP(62B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;NSFST17-EGFP(214C)/+;tdT-SNAP(62B)/+</td>
</tr>
<tr>
<td>NSF and SNAP</td>
<td>EGFP-NSF, SNAP-tdTomato</td>
<td>Larva</td>
<td>w Appl-GAL4/y;;EGFP-NSF(39B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;SNAP-tdT(253B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4/y;;EGFP-NSF(39B)/SNAP-tdT(253B)</td>
</tr>
<tr>
<td>NSF and SNAP</td>
<td>EGFP-NSF17, SNAP-tdTomato</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSFST17(123A)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;SNAP-tdT-SNAP(253B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSFST17(123A)/SNAP-tdT(253B)</td>
</tr>
<tr>
<td>SNAP and SYX</td>
<td>EGFP-SNAP, tdTomato-SYX</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;EGFP-SNAP(123C)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;tdT-SYX(196A) Δ229/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;EGFP-SNAP(123C)tdT-SYX(196A) Δ229</td>
</tr>
<tr>
<td>SNAP and NSYB</td>
<td>EGFP-NSYB, SNAP-tdTomato</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSYB(28A)ΔF33B/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;SNAP-tdT(253B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSYB (28A)ΔF33B/SNAP-tdT (253B)</td>
</tr>
<tr>
<td>SNAP and SNAP-25</td>
<td>EGFP-SNAP25, SNAP-tdTomato</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;EGFP-SNAP25(107B) MX124/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;SNAP-tdT(253B)/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;EGFP-SNAP25(107B) MX124/SNAP-tdT(253B)</td>
</tr>
<tr>
<td>NSYB and SNAP-25</td>
<td>EGFP-NSYB, tdTomato-SNAP25</td>
<td>Larva</td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSYB(28A) ΔF33B/tdT-SNAP25(236C) MX124/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;tdT-SNAP25(236C) MX124/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>w Appl-GAL4 comtST17/y;EGFP-NSYB(28A) ΔF33B/tdT-SNAP25(236C) MX124</td>
</tr>
</tbody>
</table>

* Genotypes include names of transgenic insertion lines in parentheses (named by the Ordway Lab). Δ229, ΔF33B, and MX124 are loss-of-function mutant alleles of SYX, NSYB and SNAP-25 respectively. Abbreviations: Dev.: Developmental; tdT: tdTomato.
4.3.4 FRET analysis based on Donor Dequenching

4.3.4.1 Image acquisition

Donor dequenching FRET measurement was carried out with the same imaging system. Specifically, EGFP (the donor fluorophore) was excited by an Ar 488 nm laser and emission was collected through the HQ520/40 (green) filter. Excitation and photobleaching of tdTomato was achieved through a He-Ne 543 nm laser and emission was collected through the HQ 610/75 (red) filter.

Donor dequenching measurements were carried out at adult *Drosophila* presynaptic boutons on the surface of the DLM. Presynaptic boutons co-expressing both EGFP (donor) and tdTomato (acceptor) fusion proteins were measured individually for FRET efficiency ($E$). And 16 to 27 boutons sampled from 3 adult preparations were averaged to produce the mean FRET efficiency in this study.

FRET efficiency relates quantum yields of donor fluorophore as follows,

$$E = 1 - \frac{F_{DA}}{F_D}$$

where $F_{DA}$ and $F_D$ are donor quantum yield with and without an acceptor. As discussed in the introduction of this chapter, $F_{DA}$ and $F_D$ can be experimentally determined by measuring the donor fluorescence intensity before and after the acceptor is photobleached to block energy transfer.

Selected presynaptic boutons were surrounded by a 12×12 μm square region. Prior to photobleaching, the region was scanned with the 488 nm laser and emission through the green filter was collected to produce Image1. Then the region was scanned with the 543 nm laser and emission through the red filter was collected to produce Image2. After that, the region was successively scanned 10 times with the 543 nm laser at maximum power to photobleach tdTomato molecules. Finally, Image3 and Image 4 were acquired under the same imaging configurations used for Image1 and 2, respectively.

4.3.4.2 Image processing and data analysis

Background subtraction was performed as described earlier for the 3-Cube FRET measurement. Whole bouton fluorescence intensities through the green channel before and after photobleaching were measured as $F_{DA}$ and $F_D$ from Image 1 and 3, respectively. Fluorescence intensities through the red channel before and after photobleaching, $F_R$ and $F_R'$, were measured from Image 2 and 4, respectively.

$F_{DA}$ and $F_D$ were brought into Equation 9 to calculate the FRET efficiency while $F_R$ and $F_R'$ were used to estimate the percentage photobleaching of tdTomato. Note that the inability to photobleach every acceptor molecule present is a source of error in acceptor photobleaching FRET measurements. The above photobleaching paradigm typically reduced tdTomato signal to 2-5% of original, which indicates the range of error. Repeated scanning of the 543 nm laser
produced minimal excitation/photobleach of EGFP molecules, which was ignored during data processing.

Although tdTomato was reported to mature at a fast rate (Campbell et al., 2002; Shaner et al., 2004), immature populations of tdTomato produced by transgenic expression in the *Drosophila* nervous system were observed at both larval and adult stages. As for DsRed, maturation of Tomato proceeds through a green fluorescent intermediate to reach full red fluorescence (Baird et al., 2000). Because tdTomato is composed of 2 tandem Tomato molecules, a small component of intramolecular FRET between immature and mature green and red proteins within the same dimer is predicted and was observed in our experiments. This artifact was converted by imaging tdTomato alone under the same experimental paradigm. As described above, whole bouton fluorescence through the green and red channels before and after photobleaching was measured as $F_G, F'_G, F_R$ and $F'_R$ respectively. Absolute changes of fluorescence emission through the green and red channel due to photobleaching, $\Delta G$ and $\Delta R$, was then calculated as follows,

$$\Delta G = |F_G - F'_G|$$
$$\Delta R = |F_R - F'_R|$$

The ratio of $\Delta G$ and $\Delta R$, $\alpha = \frac{\Delta G}{\Delta R}$, was used to eliminate the contribution of intramolecular FRET to the final measurement of FRET efficiency as follows,

$$E = 1 - \frac{F_D}{F_D - \alpha \times \Delta R}$$

Using soluble tdTomato expressed at nerve terminals, $\alpha$ was experimentally determined to be 0.04 at 20°C and 0.05 at 33°C. These values do not seem to vary with the concentration or cellular localization of the fluorophore. FRET pairs examined through the donor dequenching method and genotypes of experimental animals are listed in Table 4.4.

### Table 4.4 FRET pair examined with the donor-dequenching measurement.

<table>
<thead>
<tr>
<th>Binding Partners</th>
<th>FRET Pair</th>
<th>Dev. Stage</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF and SNAP</td>
<td>NSF-EGFP, tdTomato-SNAP</td>
<td>Larva</td>
<td>w Appl-GAL4/y;tdt-SNAP (100A)/+; NSF-EGFP(188A)/+</td>
</tr>
<tr>
<td></td>
<td>NSFST17-EGFP, tdTomato-SNAP</td>
<td>Larva</td>
<td>w Appl-GAL4 comST17/y;NSFST17-EGFP(214C)/tdTomato-SNAP(100A)</td>
</tr>
</tbody>
</table>

* Genotypes include names of transgenic insertion lines in parentheses (named by the Ordway Lab). Abbreviation: Dev.: Developmental.

### 4.4 Results and conclusions

#### 4.4.1 Detection of *in vivo* FRET at *Drosophila* larval and adult neuromuscular synapses.

To test if *in vivo* interaction among synaptic proteins produces FRET, the 3-Cube FRET measurement was performed at *Drosophila* third-instar larval and adult DLM neuromuscular...
synapses as described in 4.3.4. EGFP-tagged dNSF1 (dNSF1-EGFP) and tdTomato tagged dSNAP (tdTomato-dSNAP) were co-expressed in neurons as the donor and acceptor respectively. Because inactivation of dNSF1 impairs disassembly of the 20S fusion particle and could possibly result in enhanced binding of dNSF1 and dSNAP, expression of the transgenic proteins was carried out in both a wild-type (WT) and comtoseST17 (comtST17) mutant background. In comtST17, instead of dNSF1-EGFP, its mutant version, dNSF1ST17-EGFP, was used as the donor. Incubation of comtST17 preparations at the permissive temperature of 20 °C does not affect activity of dNSF1, while a short exposure to the restrictive temperature of 33 °C rapidly disrupts function of the entire dNSF1 population, including endogenous dNSF1ST17 and dNSF1ST17-EGFP. In wild type, dNSF1 is active at both temperatures.

Results of FRET measurement at larval and adult neuromuscular synapses were in good agreement with each other. As shown in Figure 4.3, FRET signals were detected at mutant synapses incubated at 33 °C. The FR measured for larva and adult were 1.1 ± 0.01 (n=16) and 1.1 ± 0.02 (n=31) respectively. Note that because transgenic lines of different expression levels were used for larval and adult experiments, these FR do not reflect relative strength of in vivo binding interaction at the two types of synapses (discussed later). In contrast, no FRET was detected at wild type synapses or mutant synapses incubated at 20 °C.

Figure 4.3 Detection of in vivo FRET using 3-Cube FRET at Drosophila third-instar larval and adult DLM neuromuscular synapses. Using EGFP-dNSF1ST17 and tdTomato-dSNAP as the donor and acceptor respectively, 3-Cube FRET detections at larval (grey) and adult (black) neuromuscular synapses were in good agreement with each other: FRET was detected in comtST17 at 33 °C, and the FR measured for larva and adult were 1.1 ± 0.01 (n=16) and 1.1 ± 0.02 (n=31) respectively. FRET was not detected in the rest conditions, and the FR measured for larva were (in the order of comtST17 20 °C, WT 20 °C, WT 33 °C) 1.0 ± 0.01 (n=12) 1.0 ± 0.02 (n=16), 1.0 ± 0.02 (n=14), and for adult were 1.0 ± 0.03 (n=17), 1.0 ± 0.02 (n=16), 1.0 ± 0.02 (n=17). Using 2-tailed t-test, FR measured for comtST17 at 33°C was proven to be significantly greater than those measured for comtST17 at 20 °C and wild type at 33 °C (p<0.001, indicated by triple asters or p<0.01, indicated by double asters).
4.4.2 Detection of *in vivo* FRET using 3-Cube and Donor Dequenching FRET measurements.

4.4.2.1 Consistency between 3-Cube and donor dequenching FRET methods

The study has employed two intensity-based methods for FRET measurement to ensure the validity and accuracy of results. 3-Cube FRET is a sensitized emission method that measures changes in acceptor emission due to FRET, whereas donor dequenching FRET is an acceptor photobleaching method that measures changes in the donor emission due to FRET (see 4.1). To test if the two methods produce consistent results within the current experimental system, EGFP-tagged dNSF1 (dNSF1-EGFP) and tdTomato tagged dSNAP (tdTomato-dSNAP) were co-expressed in the nervous system of *Drosophila* as the donor and acceptor, respectively, and both methods were used to detect FRET at adult DLM neuromuscular synapses. For analysis in a *comt*<sup>ST17</sup> mutant background, the mutant version dNSF1<sup>ST17</sup>-EGFP was expressed as the donor.

FRET detection with the two methods produced consistent results. As shown in Figure 4.4, both methods detected FRET from *comt*<sup>ST17</sup> mutant preparations, whereas neither method detected FRET from wild type preparations. Moreover, stronger FRET was detected in *comt*<sup>ST17</sup> at 33 °C as compared to 20 °C. In *comt*<sup>ST17</sup>, the FR given by 3-Cube measurement was 1.1 ± 0.02 (n=18) at 20 °C and 1.3 ± 0.03 (n=36) at 33 °C. The E given by donor dequenching measurement was at 0.1 ± 0.03 (n=16) at 20 °C and 0.2 ± 0.02 (n=26) at 33 °C.
Figure 4.4 Detection of *in vivo* FRET using the 3-Cube FRET and donor dequenching methods. FRET analysis was performed at *Drosophila* adult DLM neuromuscular synapses. Using EGFP-dNSF1<sup>(ST17)</sup> and tdTomato-dSNAP as the donor and acceptor respectively, FRET detection using the 3-Cube FRET (grey) and donor dequenching (black) measurements were in good agreement with each other. FRET was detected in *comt<sup>ST17</sup>* but not in wild type. In *comt<sup>ST17</sup>*, stronger FRET signals were detected at the restrictive temperature of 33 °C as compared to at the permissive temperature of 20 °C. *FR* given by 3-Cube FRET were (in the order of *comt<sup>ST17</sup>* 20 °C, *comt<sup>ST17</sup>* 33 °C, WT 20 °C, WT 33 °C), 1.1 ± 0.02 (n=18), 1.3 ± 0.03 (n=36), 1.0 ± 0.02 (n=19) and 1.0 ± 0.02 (n=33), and *E* given by donor-dequenching FRET were 0.1 ± 0.03 (n=16), 0.2 ± 0.02 (n=26), 0.0 ± 0.03 (n=20) and 0.0 ± 0.03 (n=23). Using 2-tailed t-test, *FR* or *E* measured for *comt<sup>ST17</sup>* at 33 °C was proven to be significantly greater than that measured for *comt<sup>ST17</sup>* at 20 °C and wild type at 33 °C (p<0.001, indicated by triple asters or p<0.05, indicated by a single aster). However, *FR* or *E* measured for *comt<sup>ST17</sup>* at 20 °C was not significantly different from that measured for wild type at 20 °C.
4.4.2.2 Detection sensitivity of 3-Cube and donor dequenching FRET measurements

Sensitivity of FRET detection by intensity-based methods is greatly affected by in vivo concentrations of fluorophores (see 4.1), a satisfying detection sensitivity relies on a high signal-to-noise ratio as well as a suitable ratio of donor and acceptor molecules. To achieve these conditions, different transgenic lines were screened for neuronal expression levels through an imaging-based procedure (see 2.4.2). Lines were first selected for low autofluorescence background to minimize influence of noise and then categorized into groups of high, medium and low expression so that the donor-to-acceptor ratios at nerve terminals can be tuned by coupling proper donor and acceptor expressing lines.

Theoretically, 3-Cube FRET is more sensitive when the donor is expressed in excess relative to the acceptor, whereas the donor dequenching FRET is optimized when the acceptor is in excess. This idea has been tested with the current experimental system. Because FRET has been detected between dNSF1-EGFP and tdTomato-dSNAP at adult neuromuscular synapses (see 4.4.2.1), the same FRET pair and experimental paradigm was used to examine the effect of donor-to-acceptor ratio on detection sensitivity of the methods. Specifically, the same donor line used in 4.4.2.1, dNSF1^[ST17]-EGFP 214C, was coupled with two acceptor lines. The line of tdTomato-dSNAP 100A, which was used in 4.4.2.1, has a significantly higher expression level, and thus produces a much lower donor-to-acceptor ratio than the line of tdTomato-dSNAP 62B. These results confirmed the theoretical prediction. As shown in Figure 4.5, the 3-Cube FRET detection is more sensitive (detects a stronger FRET signal) with a higher donor-to-acceptor ratio (established with Line 62B). The FR measured for 62B and 100A were 1.3 ± 0.03 (n=36) and 1.1 ± 0.02 (n=31) respectively, which were significantly different from each other (p<0.01). In contrast, the donor dequenching FRET detection is more sensitive with a lower donor-to-acceptor ratio (established with Line 100A). The E measured for 62B and 100A were 0.0 ± 0.01 (n=12) and 0.2 ± 0.02 (n=26) respectively, which were significantly different from each other (p<0.001).
Figure 4.5 Detection sensitivity of the 3-Cube FRET and donor dequenching measurements. FRET analysis was performed at *Drosophila* adult DLM neuromuscular synapses in a *comi*<sup>ST17</sup> mutant background at 33 °C. The donor line, dNSF<sup>ST17</sup>-EGFP (214C) were coupled with two acceptor lines, tdTomato-dSNAP 62B and 100A respectively, to establish proper donor-to-acceptor ratios at synaptic terminals. 3-Cube FRET detection was more sensitive with a higher donor-to-acceptor ratio. *FR* measured for Line 62B and 100A were 1.3 ± 0.03 (n=36) and 1.1 ± 0.02 (n=31) respectively, which were significantly different from each other (p<0.01). In contrast, the donor dequenching FRET detection was more sensitive with a lower donor-to-acceptor ratio. *E* measured for Line 62B and 100A were 0.0 ± 0.01 (n=12) and 0.2 ± 0.02 (n=26) respectively, which was significantly different from each other (p<0.001).
4.4.3 *In vivo* interaction among SNAREs, NSF and SNAP characterized by FRET

FRET has been applied to investigate the *in vivo* arrangements of subunits of the 20S fusion particle. The structure of 20S particle formed *in vitro* with purified subunits has been described in great detail on several ultrastructural studies. According to Hohl *et al.*, the cis-SNARE complex is a 2.5×15 nm rod, of which the entire length is ensheathed by α-SNAP. Hexameric NSF is a halocylinder which measures roughly 9 nm in height. When SNAREs, NSF and SNAP assemble together, the resulting 20S fusion particle measures 22 nm in length and varies in width from 6nm to 13.5 nm (Hohl *et al.*, 1998b). Because FRET is a short range effect with an effective distance of no longer than 10 nm, it may occur between subunits only when fluorophores are attached to protein termini that are positioned close to each other in the 20S particle. Furthermore, FRET produced by a same pair of interacting subunits with fluorophores attached to different termini may vary significantly in signal robustness, which may be informative with regard to the *in vivo* subunit arrangement of the 20S particle.

4.4.3.1 Orientations of NSF and SNAP in assembly within the 20S particle

To examine the *in vivo* orientation of dNSF1 and SNAP in assembly with each other, dNSF1 and SNAP with an N- or C-terminal fluorescent tag were co-expressed at neuromuscular synapses of *Drosophila* third-instar larvae. 3-Cube FRET analysis was performed to examine FRET produced by the EGFP-dNSF1 and dSNAP-tdTomato FRET pair and the dNSF1-EGFP and tdTomato-dSNAP FRET pair. Again, for analysis in the *comatose* mutant background, EGFP tagged dNSF1<sup>ST17</sup> was used as the donor. As shown in Figure 4.6, for both pairs, FRET was detected only in *comt<sup>ST17</sup>* at the restrictive temperature, with the EGFP-dNSF1 and dSNAP-tdTomato pair producing a significantly stronger signal. The FR measured for the two pairs were 1.1 ± 0.01 (n=16) and 1.2 ± 0.01 (n=32) respectively, which were significantly different from each other (p<0.001). This result suggests that the N-terminus of dNSF1 and the C-terminus of dSNAP maybe in closer proximity when assembled in 20S particles. This is consistent with *in vitro* structural studies of 20S particles (Figure 1.8) as well as the proposed function of the dNSF1 N-terminal domain in associating with SNAP/SNAREs and the α-SNAP C-terminal domain in binding and activating NSF (see more in introduction). In this study, donor and acceptor lines with similar expression levels were selected for both pairs to minimize their influence on FRET detection and comparison. However, it’s hard to exclude that the stronger signal produced by one pair is due to more sensitive FRET detection under the specific situation.
Figure 4.6 *In vivo* orientation of dNSF1 and dSNAP in assembly with each other suggested by FRET. 3-Cube FRET was performed at neuromuscular synapses of *Drosophila* third-instar larvae in both wild-type and *comt*<sup>ST17</sup> mutant background. FRET detection for the pair of dNSF1<sup>(ST17)</sup>-EGFP and tdTomato-dSNAP (grey) was compared with the pair of EGFP-dNSF1<sup>(ST17)</sup> and dSNAP-tdTomato (black). For both pairs, FRET was only detected in *comt*<sup>ST17</sup> at the restrictive temperature of 33 °C, with a stronger signal detected for the pair of EGFP-dNSF1<sup>(ST17)</sup> and dSNAP-tdTomato, which indicated a closer proximity of the N-terminus of dNSF1 and C-terminus of dSNAP in assembly with each other. The FR measured for the two pairs were 1.1 ± 0.01 (n=16) and 1.2 ± 0.01 (n=32) respectively, which were significantly different from each other (p<0.001). In the rest conditions, FR measured for the pair of dNSF1<sup>(ST17)</sup>-EGFP and tdTomato-dSNAP were (in the order of comtST17 20C, WT 20C, WT 33C) 1.0 ± 0.01 (n=12), 1.0 ± 0.02 (n=16) and 1.0 ± 0.02 (n=14), and FR measured for the pair of EGFP-dNSF1<sup>(ST17)</sup> and dSNAP-tdTomato were 1.0 ± 0.01 (n=19), 1.0 ± 0.01 (n=16) and 1.0 ± 0.01 (n=17). And no statistically significant difference between the two pairs was confirmed by 2-tailed t-test.
4.4.3.2 *In vivo* interactions between SNAP and SNAREs characterized by FRET analysis

To investigate *in vivo* interactions between SNAP and its membrane receptors, SNAREs, using FRET, EGFP was fused to the N-terminus of SNAREs, which is the cytosolic end of SYX and n-syb and the membrane distal end of SNAP-25, and tdTomato was tagged on either the N- or C-terminus of dSNAP. Because accumulation of the 20S fusion particle upon inactivation of dNSF1 could possibly result in enhanced binding interaction between SNAREs and dSNAP, SNARE and dSNAP were co-expressed at neuromuscular synapses of third-instar larvae in a *comtST17* mutant background and FRET studies were carried out at both permissive 20 °C and restrictive 33 °C using the 3-Cube FRET method.

First, interactions between EGFP-SNAREs and tdTomato-dSNAP were examined and FRET was not detected for any of the pairs (data not shown). Then FRET was measured for EGFP-SNAREs and dSNAP-tdTomato. And as shown in Figure 4.7, FRET was detected only for the EGFP-SNAP-25 and dSNAP-tdTomato pair with a more robust signal detected at 33 °C as compared to 20 °C. The FR was 1.1 ± 0.02 (n=16) at 20 °C and 1.2 ± 0.04 (n=14) at 33 °C, which were significantly different from each other (p<0.05).

![Figure 4.7 In vivo interactions between SNAREs and SNAP characterized by FRET.](image)

**Figure 4.7 In vivo interactions between SNAREs and SNAP characterized by FRET.**

3-Cube FRET analysis was performed at neuromuscular synapses of *Drosophila* third-instar larvae in a *comtST17* mutant background. Acute inactivation of *in vivo* dNSF1*ST17* function was achieved by temperature shifting from the permissive 20 °C to the restrictive 33 °C. FRET detections for the pair of N-terminal EGFP tagged SNARE (NSYB, SNAP-25 or SYX) and dSNAP-tdTomato at both temperatures were compared with each other. FRET was detected for the pair of EGFP-SNAP-25 and dSNAP-tdTomato with a stronger signal detected at 33 °C, which suggests a direct binding of dSNAP to the SNARE core complex via interaction with SNAP-25. The FR measured for 20 °C and 33 °C were 1.1 ± 0.02 (n=16) and 1.2 ± 0.04 (n=14), which were significantly different from each other (p<0.05). No FRET was detected between dSNAP and NSYB or SYX. The FR measured for dSNAP and NSYB were 1.0 ± 0.03 (n=4) at 20 °C and 1.0 ± 0.02 (n=4) at 33 °C. The FR measured for dSNAP and SYX were 1.0 ± 0.03 (n=4) at 20 °C, and 1.0 ± 0.02 (n=7) at 33 °C.
4.5 Discussion

4.5.1 Significance of the current FRET study

Using the Drosophila neuromuscular synapse as the model system, the current study has demonstrated successful detection of in vivo FRET produced by neuronal protein interactions at living synapses and has further probed the in vivo mechanism underlying synaptic vesicle trafficking. To ensure validity and accuracy of results, these studies have employed two independent FRET methods, 3-Cube and Donor Dequenching, and consistency between the two has been confirmed experimentally. Moreover, the two methods are suitable for FRET analysis under different conditions, and thus complement each other. This is particularly important in the current study, because neuronal expression of fluorescent fusion proteins was achieved through use of the UAS-GAL4 system and the expression levels couldn’t be controlled precisely. FRET analysis using the above detection methods has helped to gain insights into the in vivo properties of binding interactions among synaptic proteins, including conserved properties at the larval and adult developmental stages, arrangements and orientation of proteins within complexes and activity-dependent dynamics of association during synaptic transmission (see chapter 3).

4.5.2 Interpretation of negative results

A general rule applies when interpreting FRET results of the current study. That is, while positive FRET detection can be considered an indication of a protein binding interaction, negative FRET results (no FRET) do NOT prove the absence of binding. This is because successful FRET detection within the current experimental system depends on many factors, including those affecting detection sensitivity of intensity-based measurements discussed earlier. Other factors which may have caused negative FRET detection in this study will be discussed below.

4.5.2.1 Negative FRET detection between dNSF1 and dSNAP under wild-type conditions

A major effort of the current FRET study has been applied to investigate in vivo properties of binding interactions between dNSF1 and dSNAP. Specifically, FRET analysis was performed with both the 3-Cube and Donor Dequenching methods and was carried out in both larval and adult developmental stages. Moreover, orientations of the proteins in assembly with each other have been examined by appending fluorescent tags to different ends of the proteins. Notably, all the analyses have produced the consistent result that FRET was detected when dNSF1 activity was disrupted in comatose mutants at the restrictive temperature, whereas no FRET signal was detected under the wild-type conditions. However, as discussed earlier, disassembly of SNARE complexes through binding of dNSF1 and dSNAP is indispensable for maintaining local SNARE protein recycling at synapses and thus synaptic functions under normal (wild-type) conditions. This may be explained by the inability of current FRET detection methods to capture transient in vivo protein binding interactions during normal cycling processes. In fact, the positive FRET detection in comatose luckily results from a stalled assembly-disassembly cycle of the 20S fusion particle at the post-fusion stage and its subsequent accumulation following inactivation of dNSF1. The same interpretation may explain the enhanced FRET between EGFP-SNAP-25 and dSNAP-tdTomato in comatose at
33 °C as compared to 20 °C.

### 4.5.2.2 Negative FRET detection between SNAREs and dSNAP

To investigate in vivo interactions between SNAP and its membrane receptors, SNAREs, the donor fluorophore EGFP was selectively fused to the N-terminal ends of SNAREs. This is because the C-termini of SNAREs form the membrane anchor of the core complex, and membranes could become a physical obstruction that prevents FRET (especially for n-syb and SYX whose C-termini are located across the membrane). When the acceptor fluorophore tdTomato was appended to the N-terminus of dSNAP, FRET was not detected between dSNAP and any of the SNARE proteins. Several lines of evidence suggest that α-SNAP ensheaths the entire length of the SNARE core complex in an anti-parallel fashion, which positions its C-terminal end in a closer proximity to N-termini of SNAREs (Hanson et al., 1997; Wimmer et al., 2001). Taking into consideration the dimensions of the core complex (see 2.4.3), the distance between EGFP and tdTomato could still go beyond the effective distance of FRET (about 10 nm) after binding of dSNAP. Moreover, EGFP was fused to the N-terminal end of the SYX Habe domain, which is displaced away when the H3 domain participates in forming the coiled-coil bundle of SNARE core complex. Therefore, it’s not surprising that FRET was not detected between EGFP-SYX and dSNAP-tdTomato. Notably, robust FRET was detected between the N-terminal tagged SYX and Rop, suggesting that the N-terminal domains of SYX are actively involved in binding interactions with the soluble regulator factor Rop (Kawasaki and Ordway, 2007). Finally, the absence of FRET between EGFP-nsyb and dSNAP-tdTomato may reflect that the vast majority of EGFP-nsyb resides on synaptic vesicles in the reserve vesicle pool and does not form complexes with other SNAREs or dSNAP.

### 4.5.3 Biological meaning of results from the current FRET study

#### 4.5.3.1 In vivo evidence for the accumulation of 20S fusion complex containing SNAREs, dNSF1 and dSNAP

Through years of study, a 20S fusion complex containing SNAREs, NSF and SNAP has been found to be at the core of synaptic vesicle fusion. According to the classic model of SNARE-mediated membrane fusion, assembly of SNAREs into the ternary core complex catalyzes the priming and fusion reactions of docked synaptic vesicles. After fusion, The ATPase NSF and its co-factor SNAP catalyze the conformational change and subsequent disassociation of SNARE complexes via ATP hydrolysis. This is essential for maintaining synaptic activity because free SNAREs are released for future fusion events during this process. Accumulation of the ternary SNARE core complex when NSF function is disrupted has been shown prior to this study. Using the Drosophila larval neuromuscular synapses as the model system, the current FRET analysis has demonstrated enhanced in vivo interactions between dNSF1 and dSNAP and between SNAP and SNAP-25 at the restrictive temperature of comatose, and thus support the proposed model of dNSF1 and dSNAP accumulation with the SNARE core complex in the 20S fusion particle following inactivation of dNSF1 (see 3.3.1).
4.5.3.2 In vivo evidence for the arrangement of subunits in 20S fusion particles

*In vitro* investigations of the assembly and arrangement of subunits within the 20S fusion particle using purified proteins have been particularly successful in revealing detailed structures of the complex (see 1.2.2.4). In the current FRET study, some supporting *in vivo* evidence has been generated by detecting and comparing signal robustness of FRET produced by interacting subunits with the donor and acceptor fluorophores attached to different ends of each protein.

Specifically, EGFP-dNSF1 and dSNAP-tdTomato produced significantly stronger FRET as compared to FRET between dNSF1-EGFP and tdTomato-dSNAP under same experimental conditions (see 4.4.3.1), which suggests closer proximity between the N-terminus of dNSF1 and C-terminus of dSNAP. This is consistent with the orientations of dNSF1 and dSNAP in assembly with each other as suggested by *in vitro* ultrastructural studies and also agrees with the proposed domain functions of the proteins. Similarly, EGFP-SNAP-25 and dSNAP-tdTomato produced robust FRET whereas no FRET was detected between EGFP-SNAP-25 and tdTomato-dSNAP (see 4.4.3.2). This suggests that the distance between the N-termini of SNAP-25 and dSNAP could exceed the effective range of FRET (about 10 nm) and is consistent with the proposed lateral, anti-parallel binding of SNAP to the SNARE core complex in a sheathed manner.

4.5.3.3 Conserved dNSF1 function at larval and adult developmental stages

Electrophysiological and ultrastructural studies demonstrated progressive, activity-dependent reduction in neurotransmitter release and a marked accumulation of docked synaptic vesicles in *comatose* under the restrictive condition, which has suggested a role for dNSF1 in mediating priming reactions of synaptic vesicles (Kawasaki *et al.*, 1998). Further, the molecular nature of this priming role has been shown to involve NSF-dependent disassembly of SNARE complexes downstream of vesicle docking (Tolar and Pallanck, 1998). In a more recent study, electrophysiological evidence were combined with *in vivo* imaging analysis, and has provided evidence that dNSF1 serves to maintain free t-SNAREs available for vesicle priming at presynaptic active zones(Kawasaki and Ordway, 2009). So far, studies of the *comatose* mutation have been carried out in adult *Drosophila*. However, the current study has also employed the larval neuromuscular synapse, and has found consistency with the proposed model of dNSF1 function (see Chapter 3). Further, in the current FRET study, binding interactions between dNSF1 and its cofactor dSNAP were examined at both larval and adult synapses. Results of analysis performed at the two developmental stages were in good agreement with each other (see 4.4.1), which also suggests similar functional properties of dNSF1 throughout development.

However, larval neuromuscular synapses exhibit several distinct properties from that of adults. First, distributions of dNSF1 at larval and adult neuromuscular synapses differ, although some similarity is observed between the two. Specifically, distribution of dNSF1 at adult synapses is less diffuse with enrichment near presynaptic active zones (data not shown). Moreover, TS mutations of *comatose* exhibit distinct electrophysiological phenotypes at the two developmental stages. Specifically, the fast activity-dependent synaptic depression of
adult synapses is not observed in larvae (data not shown). Additionally, loss-of-function mutations of *comatose* lead to lethality at the pharate adult stage, which suggests that neuronal activity of dNSF1 is not required at the larval stage. These observations suggest immature synaptic properties of larval synapses as a developmental intermediate, and also raise the interesting possibility that loss of dNSF1 function at the larval state could be compensated by the other *Drosophila* NSF homolog, dNSF2, which exhibits 84% amino acid identity to dNSF1 (Boulianne and Trimble, 1995; Pallanck *et al*., 1995b).

FRET was sometimes detected between dNSF1 and dSNAP in *comatose* under the permissive condition, although these signals were usually weak and not statistically different from background noises (Figure 4.4). Also, slightly altered bouton morphologies were sometimes observed in *comatose* larvae raised at the permissive temperature (data not shown). These observations suggest that the *comatose* mutation may not be completely conditional and could possibly affect dNSF1 function throughout development.
Chapter 5  Discussion

5.1 Implications of the project

5.1.1 A model for spatial arrangement of protein functions with respect to presynaptic active zones

This project has been focused on in vivo functions and interactions among SNARE proteins, SNAP and NSF, which have been shown to play essential roles in synaptic vesicle trafficking. A primary focus has been on NSF, which functions to disassemble SNARE complexes downstream of vesicle fusion. Taking advantage of the Drosophila genetic model system and the TS dNSF1 mutant, comatose, this project has combined biochemical, immunocytochemical and in vivo live imaging approaches and led to several interesting discoveries which advance our understanding of the behavior and interactions of presynaptic proteins at native synapses. The results reveal a spatial arrangement of SNARE, dSNAP and dNSF1 functions and interactions with respect to the presynaptic active zone, and strongly support a model in which post-fusion SNARE complexes move out of active zones to be disassembled within the PAZ regions of the presynaptic plasma membrane by dNSF1 and dSNAP. After disassembly, active zones are replenished with free t-SNAREs competent for vesicle fusion and free v-SNAREs are incorporated into nascent synaptic vesicles. Meanwhile, following disassembly of SNARE complexes, dNSF1 and dSNAP disassociate from the plasma membrane and return to the cytosol.

5.1.2 Live Imaging, an important research methodology in cell biology

Ever since the earliest examination of cellular structures, biologists have been fascinated by examining the properties of cells through microscopy. At present, live cell imaging has become a requisite technology for cell biology research because it provides the exciting opportunity to observe processes as they happen within the cell and adds a vital extra dimension to the understanding of cell function. The rapid advance in fluorescent protein technology has allowed expression of genetically encoded fluorescent proteins in cells and organisms and thus permitted time lapse imaging of fluorescent proteins in living cells. This approach allows observation of dynamic properties of fluorescent fusion proteins for extended time periods and quantitative analysis of living cells. For example, monitoring protein dynamics through time-lapse FRAP analysis and quantifying protein interactions with FRET measurements (Section 5.1.3) have provided critical insights into in vivo activities of synaptic proteins in the current study.

To study the molecular mechanisms of synaptic function and physiology, the ability to address research questions in living animals is very important. The combination of live imaging with the model system of Drosophila neuromuscular synapses has been a key aspect of this project. The system exhibits surprisingly high tolerance of transgenic expression of fluorescent fusion proteins. Most fluorescent fusion proteins distribute normally at synapses just as their endogenous counterparts and are able to rescue mutations within the corresponding endogenous genes. One disadvantage of live imaging as compared to imaging fixed preparations is the lack of markers to identify important synaptic structures such as synaptic plasma membranes, active zones and vesicle clusters. However, this can be
overcome by performing dual or multiple color imaging. In fact, fluorescently tagged presynaptic calcium channel protein, cacophony, has been used as a marker for active zones in live imaging studies.

5.1.3 In vivo FRET analysis

Investigation of protein interactions using FRET has become a commonly used approach in cell biology. FRET is a short range effect that occurs only over a distance of a few nanometers and therefore provides an accurate and informative indication of direct protein interactions. Moreover, FRET analysis allows the study of protein interactions in vivo. In this project, FRET methods developed and adapted in our laboratory have been employed in the Drosophila model system, to directly monitor binding interactions of synaptic proteins at native neuromuscular synapses. 3-Cube FRET is the primary method used in the project, which exhibits satisfiable sensitivity and confidence of detection. A complementary FRET method, Donor Dequenching, has been used as well. Both methods produce consistent results within the experimental system, and provide increased confidence in the FRET analysis. Taking advantage of the FRET assay, this project reveals interesting properties of in vivo binding interactions among SNAREs, dSNAP and dNSF1 during synaptic vesicle trafficking and provides important evidence in support of the model discussed in 5.1.1.

Importantly, the in vivo FRET assay was able to detect changes in protein interaction elicited by synaptic activity. An activity-dependent increase in dNSF1 and dSNAP interaction was observed along with their activity-dependent redistribution to the PAZ. The exciting possibility of monitoring and quantifying dynamic cellular processes is especially valuable for investigation of synaptic mechanisms and serves as an important complement to studies of synaptic protein functions and interactions using biochemical and electrophysiological approaches.

5.2 Future Directions

5.2.1 Identification of binding partners for dNSF1 at the PAZ

One open question is related to the mechanism underlying immobilization of dNSF1 at the PAZ after its activity-dependent redistribution. To identify interacting proteins of dNSF1 at the PAZ, one approach is to selectively examine known binding partners of NSF. For example, this study strongly suggests association of dNSF1 with dSNAP and SNAREs at the PAZ. To see if association in 20S complexes might explain the low mobility of redistributed dNSF1, a reasonable question to ask is whether SNAREs are also stably associated with the PAZ under the same experimental condition. Also, efforts have been made to examine whether immobilization of dNSF1 is mediated by its association with cytoskeleton components. Preliminary studies using latrunculin A to depolymerize actin filaments did not prevent immobilization of dNSF1 at the PAZ, suggesting that actin is not an important determinant in dNSF1 redistribution. An alternative approach is to purify synaptic components that exhibit enhanced binding interactions with dNSF1 at a restrictive temperature in comatose. Candidate binding partners may be enriched by co-immunoprecipitation with dNSF1, recovered from a denaturing gel and identified using mass spectrometry.
5.2.2 Improve detection sensitivity of in vivo FRET

As discussed in Chapter 4, overexpression of fluorescent fusion proteins, their competition with endogenous proteins for binding partners and unfavorable donor-to-acceptor ratios may negatively affect in vivo FRET analysis. These problems may be addressed in the Drosophila genetic model system. First, transgene expression levels may be optimized through use of the TARGET system, which adds temporal control to the tight spatial control provided by transgenic expression using the traditional GAL4/UAS system (McGuire et al., 2003). Specifically, GAL80 protein normally functions as a repressor of GAL4, and its TS form, GAL80ts, can be acutely inactivated by exposure to the restrictive temperature. Therefore, in a GAL80\(^{ts}\) background, transgenic expression of fluorescent fusion proteins driven by GAL4/UAS can be induced for a brief period to avoid overexpression. Second, to minimize the influence of non-fluorescent endogenous proteins on FRET detection, FRET analysis can be performed in a genetic background homozygous for a null allele of the endogenous gene. This is made possible in some cases by transformation rescue of homozygous mutants through expression of the corresponding fluorescent fusion protein. Finally, the donor-to-acceptor ratio of a FRET pair can be controlled by tagging candidate binding partners with different numbers of donor and acceptor fluorophore peptides. For example, robustness of the 3-Cube FRET may be improved by tagging a protein with two tandem repeats of EGFP given that transgenic expression levels and binding interactions remain the same.
Bibliography


Chen MS, Obar RA, Schroeder CC, Austin TW, Poodry CA, Wadsworth SC, Vallee RB (1991)
Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. Nature 351:583-586.


Biochemistry 37:10354-10362.
Molecular Cell Biology 7:631-643.


interactions are not selective Journal of Biological Chemistry 274:5649-5653.
VITA

Wenhua Yu

EDUCATION
Ph.D. in Genetics, Pennsylvania State University, UP 2004.08-2010.08
B.S. in Pharmacy, Wuhan University, Wuhan, China 2000.09-2004.06

EXPERIENCE
Research Assistant  Pennsylvania State University  2004.10-2010.6
Teaching Assistant  Pennsylvania State University  2005-2009
Research Assistant  Wuhan University  2003.01-2004.06

PUBLICATIONS

PRESENTATIONS
