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Activity-Dependent Interactions of NSF and SNAP at Living Synapses

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Abstract

As core components of the neurotransmitter release apparatus, SNAREs, NSF and SNAPs mediate fusion of neurotransmitter-filled synaptic vesicles within specialized regions of the presynaptic plasma membrane known as active zones (AZs). The present study combines genetic approaches in *Drosophila* with biochemical and live-imaging methods to provide new insights into the *in vivo* behavior and interactions of NSF and SNAP in neurotransmitter release. This work employs a temperature-sensitive (TS) paralytic NSF mutant, *comatose*, to show that disruption of NSF function results in activity-dependent redistribution of NSF and SNAP to periactive zone (PAZ) regions of the presynaptic plasma membrane and accumulation of protein complexes containing SNAREs, NSF and SNAP. Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Recovery After Photobleaching (FRAP) studies in *comatose* revealed that NSF and SNAP exhibit activity-dependent binding to each other within living presynaptic terminals as well as distinctive interactions and mobilities. These observations extend current models describing the spatial organization of NSF, SNAP and SNARE proteins in synaptic vesicle trafficking.

1. Introduction

Previous studies identified core components of the neurotransmitter release apparatus including: N-ETHYLMALIMIDE SENSITIVE FACTOR (NSF), the SOLUBLE NSF ATTACHMENT PROTEINS (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 SNARE complexes which promote synaptic vesicle fusion and neurotransmitter release [reviewed (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 synaptic vesicles through an endocytic process.

Despite this 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

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and interactions of these proteins at living synapses. An exciting approach to this challenge is provided by the *Drosophila* model system, in which conserved mechanisms of neurotransmitter release may be investigated through a powerful combination of genetic, molecular, electrophysiological and imaging methods. Our previous work at adult Dorsal Longitudinal Muscle (DLM) neuromuscular synapses in a temperature-sensitive (TS) paralytic dNSF1 mutant, *comatose* (*comt*), has suggested a model in which NSF-mediated disassembly of SNARE complexes in the PAZ sustains an AZ pool of free t-SNAREs during synaptic activity (Kawasaki and Ordway, 2009). In the present study, live imaging of NSF and SNAP fluorescent fusion proteins in the larval neuromuscular synapse preparation extends this model by defining the behavior and interactions dNSF1 and SNAP at living synapses.

2. Materials and Methods

2.1 Fly stocks

Wild-type (*Canton S*), *comatose*^{ST17} (*comt*^{ST17}), CAC-tdTomato and *Appl-GAL4* stocks were from our laboratory collection. Transgenic expression was achieved using the GAL4-UAS system (Brand and Perrimon, 1993). The X-linked pan-neural driver, *Appl-GAL4*, was used to drive expression of UAS transgenes in a wild-type genetic background. The *Appl-GAL4 comt*^{ST17} recombinant line was generated in our laboratory and used to drive transgene expression in a *comatose* mutant background. All stocks were cultured on conventional cornmeal/molasses/yeast medium at 20 °C.

2.2 Generation of transgenic lines

Conventional methods were used to generate UAS transgene constructs for expression of EGFP-dSNAP, dSNAP-tdTomato, EGFP-dNSF1, EGFP-dNSF1^{ST17}, dNSF1-EGFP and dNSF1^{ST17}-EGFP. Typically, EGFP or tdTomato was appended to the N- or C-terminus using a 2 amino acid linker sequence which also introduced a unique restriction site. Constructs were incorporated into the transformation vector, pUAST (Brand and Perrimon, 1993), followed by generation of transgenic lines as described previously (Kawasaki et al., 2002).

2.3 Immunoprecipitation (IP) and western blotting were carried out essentially as described previously (Zou et al., 2008) with modifications. Prior to generating head homogenates, flies were exposed to 38 °C for 10-minutes. For each IP, approximately 600 heads (~60mg) were homogenized in a total volume of 600 µl of fresh ice-cold lysis buffer. After pre-clearing, IP was carried out with 500 µl of the remaining lysate, 50 µl of a 50% slurry of BSA blocked protein A- sepharose beads, and 5 µg of mouse anti-SYX antibody (mAb 8C3, DSHB Hybridoma, University of Iowa, IO) or equivalent amount of pre-immune mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Conventional western-blot analysis was performed following the IP procedure as described previously (Kawasaki et al., 2004). For analysis of pre-IP inputs and IP samples, 0.5 head/per lane and 400 heads/per lane were loaded onto a 9% gel, respectively. The following primary antibodies and dilutions were utilized: Anti-SYX mAb 8C3 (1:10); rabbit anti-NSYB (1:30) (developed in our lab); rabbit anti-SNAP-25 N-terminus (1:2,000) (kindly provided by Dr. David Deitcher, Cornell University); rabbit anti-dNSF (1:500) and rabbit anti-dSNAP (1:1,000) (kindly provided by Dr. Leo Pallanck, University of Washington). For quantitation of Western blots, ECL signal intensities were measured using a Storm 860 Imaging System (GE HeathCare, Piscataway, NJ).

2.4 Immunocytochemistry and confocal microscopy

Immunocytochemistry of *Drosophila* larval neuromuscular synapses was performed as described previously (Kawasaki et al., 2004) with modifications. For the experiments shown in Figure 2 B, C, dissected preparations were incubated at 33 °C for 2 min then subjected to 1 min of 20Hz stimulation followed by immediate fixation with warm (33 °C) fixative [4% paraformaldehyde in 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)]. Primary antibodies included a rabbit anti-dNSF antibody (1:5,000), a mouse anti-CSP antibody (1:200, kindly provided by Dr. Konrad Zinsmaier, University of Arizona) and anti-BRP mAb NC82 (1:50, kindly provided by Dr. Erich Buchner, University of Wurzburg, Germany).

2.5 Live imaging

Neuromuscular synapses of the ventral longitudinal muscles in third instar larvae were prepared as described previously (Wu et al., 2005). All images were obtained from presynaptic boutons located on Muscle 6 or 7 in abdominal segments A2 or A3. Preparations were perfused with 10 mM L-glutamic acid and 20 μM Nicardipine in saline solution to reduce muscle movement during imaging. The perfusion rate was 0.5 ml/min. For motor axon stimulation, the segmental nerve was severed and pulled into a suction electrode (Jan and Jan, 1976). A Master-8 stimulator (A.M.P.I., Israel) was used to deliver 15V stimulus pulses of 0.2 msec duration. Temperature control was achieved using a Medical Systems TC-202 temperature controller and PDMI micro-incubator (Medical Systems, Greenvale, NY). Temperature shifts from 20 to 33 °C typically required 4 min. Images were obtained using a Zeiss LSM confocal microscope (Carl Zeiss, Germany) using a Plan-Apochromat 63x 1.0 numerical aperture water immersion objective (Carl Zeiss). Excitation was achieved using Ar 488nm and He-Ne 543nm lasers. A Z488/543RPC dichroic mirror (Chroma, Brattleboro, VT) was used with HQ520/40 and HQ610/75 emission filters (Chroma). For FRET analysis, the “3-Cube” configurations were (cube, excitation, emission), EGFP, 488 nm, HQ520/40; tdTomato: 543 nm, HQ610/75; FRET, 488 nm, HQ610/75.

Quantification of activity-dependent redistribution—Correction for background fluorescence and photobleaching, as well as image subtraction, were carried out using the Arithmetic function of the Metavue software package (Universal Imaging Corporation, Downingtown, PA). Gaussian curve-fitting of histograms was performed in the data analysis program IGOR (WaveMetrics, Lake Oswego, OR).

Fluorescence recovery after photobleaching (FRAP)—A single bouton was analyzed in each experiment. To generate a bleached region, horizontal line scanning across the center of the bouton was repeated 10 times using the 488nm laser. Immediately after bleaching, a continuous time-lapse series of image frames was taken to monitor FRAP for 1 min. For analysis, Region of Interest (ROI) #1 included the entire bouton and ROI #2 was located over the bleached region. These ROIs were also duplicated and placed outside of the bouton to measure background fluorescence. Integrated fluorescence intensity values for these regions were processed as follows: Following background subtraction, both ROI#1 and ROI#2 values for each frame in the time series were normalized to those obtained before photobleaching. Next, normalized fluorescence intensity for ROI #1 was plotted against the frame number (n). These data were fit with a linear trendline and the resulting slope was used to correct for photobleaching during image acquisition. After correction for photobleaching, 0% FRAP was defined as the ROI #2 value for the first recovery image after photobleaching and 100% FRAP was defined as the average ROI #1 value for the entire time series. Finally, scaled values of ROI #2 were plotted as a function of time to produce the FRAP curve. Because SNAP exhibited a very high mobility at WT synapses

exposed to 33, it was not possible to resolve a bleached region of interest thus precluding FRAP analysis of SNAP under these conditions.

Fluorescence Resonance Energy Transfer (FRET)—3-Cube FRET images were acquired for individual presynaptic boutons and one or more FRET ratios (*FRs*) calculated for each bouton. For resting synapses, multiple boutons were sampled randomly. Mean *FRs* represented averages of at least 16 boutons from 4–5 preparations. For stimulated synapses, one bouton was sampled at specific time points before, during and after stimulation. The method for calculating *FR* was adapted from previously published work (Erickson et al., 2001; Erickson et al., 2003). Briefly, three ratios representing constant values, R_A , R_{D1} and R_{D2} , are obtained from the emission for specific cubes (S_{cube}) when the donor (D) or acceptor (A) is expressed alone in the system. These can be determined experimentally as follows:

$$\begin{aligned} R_A &= S_{\text{FRET}}(A) / S_{\text{tdTomato}}(A) \\ R_{D1} &= S_{\text{FRET}}(D) / S_{\text{EGFP}}(D) \\ R_{D2} &= S_{\text{tdTomato}}(D) / S_{\text{EGFP}}(D) \end{aligned}$$

As described previously, these constant values were used to correct for channel cross-talk due to spectral overlap of the donor and acceptor fluorophores. Finally, when both fluorophores were co-expressed (AD), the *FR* reflecting the acceptor emission increase due to FRET was calculated as:

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

Note that $FR > 1$ indicates FRET whereas $FR = 1$ is expected the absence of FRET.

2.6 Data analysis

Numerical data processing and graphing were performed in Microsoft Excel (Seattle, WA). All data values in the text and bar graphs were given as mean $\pm$ SEM. Statistical analysis was performed using the two-tailed Student's *t* test, and significance was assigned to comparisons with $p < 0.05$.

3. Results

3.1 Accumulation of protein complexes containing SNAREs, dNSF1 and dSNAP upon inactivation of dNSF1

The *Drosophila* NSF protein, dNSF1, plays an important role in synaptic vesicle trafficking and neurotransmitter release (Kawasaki et al., 1998; Kawasaki and Ordway, 1999; Kawasaki and Ordway, 2009; Littleton et al., 2001; Pallanck et al., 1995; Tolar and Pallanck, 1998). 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 SNARE complexes following disruption of dNSF1 activity (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 dSNAP. IP of *Drosophila* SYX 1A was performed on *Drosophila* head homogenates from wild-type and *comatose* flies exposed to a restrictive temperature of 38 °C for 10 minutes (Figure 1). Co-IP of SYNAPTOBREVIN (NSYB), SNAP-25, dNSF1 and dSNAP was observed, consistent with previous studies demonstrating

assembly of SNAREs, NSF and SNAPs into a 20S protein complex (Söllner et al., 1993; Wilson et al., 1992). While a similar level of SYX 1A IP was observed in *comatose* and wild-type samples (Fig. 1 A), co-IP of each protein was consistently higher in *comatose* (Fig. 1 B) indicating that acute disruption of dNSF1 function leads to accumulation of 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 (Söllner et al., 1993).

3.2 Presynaptic localization of endogenous dNSF1

To examine the spatial distribution of the preceding interactions of dNSF1 and SNAP at native synapses, the *in vivo* distribution of dNSF1 was examined in the larval neuromuscular synapse preparation. As shown in Figure 2 A, immunolabeling of wild-type synapses with a polyclonal anti-dNSF antibody revealed a dNSF1 distribution similar to that of synaptic vesicles, which occupy cytosolic regions within presynaptic boutons. This signal was eliminated in the dNSF1 null mutant, *comt^{CLP1}* (Golby et al., 2001; Sanyal and Krishnan, 2001), demonstrating that it specifically reflects the distribution of dNSF1 (not shown). These findings indicate that dNSF1 is localized primarily to the cytosolic compartment of presynaptic boutons.

3.3 dNSF1 redistributes to PAZ regions of the presynaptic plasma membrane in *comatose*

To determine the *in vivo* distribution of dNSF1 under conditions favoring its assembly with SNAP and SNARE proteins, dNSF1 localization was determined at *comatose* larval neuromuscular synapses following disruption of dNSF1 function. Wild-type and *comatose* preparations at the restrictive temperature of 33 °C were subjected to 20 Hz stimulation 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, synapses (Fig. 2 B, C). Localization of dNSF1 was determined relative to markers for synaptic vesicles (Fig. 2 B) as well as AZ regions of the presynaptic plasma membrane where synaptic vesicles dock and fuse (Fig. 2 C). Following its redistribution in *comatose*, dNSF1 exhibited a punctuate pattern which no longer resembled that of a synaptic vesicle marker (Fig. 2 k-o). Moreover, a clear relationship was observed between the punctuate pattern of redistributed dNSF1 and the AZ. dNSF1 puncta were adjacent to AZs such that the AZ marker fit nicely into dim regions of the dNSF1 distribution (Fig. 2 u-y). In contrast, the dNSF1 distribution at wild-type synapses remained similar to that of a synaptic vesicle marker (Fig. 2 f-j) and exhibited no clear relationship to AZs (Fig. 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.

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^{ST17}-EGFP, was expressed in a *comatose* (*comt^{ST17}*) mutant background. This approach permits live imaging before, during and after synaptic stimulation to define the activity-dependence of the dNSF1 redistribution observed in *comatose* (Fig. 3 A). For wild-type synapses maintained at 33 °C, 1 minute of 20 Hz stimulation had little impact on the distribution of dNSF1-EGFP, which remained similar to that of wild-type endogenous dNSF1 (*upper panels*). In contrast, the same stimulation paradigm in *comatose* produced clear redistribution of dNSF1^{ST17}-EGFP into a punctate pattern which persisted long after the stimulation train (*lower panels*). Finally, redistribution of dNSF1^{ST17}-EGFP was conditional in the *comatose* mutant as it was not observed after 20 Hz stimulation at 20 °C (not shown). 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 the corresponding Gaussian fits (Fig. 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 (Fig. 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^{ST17}-EGFP in the absence of stimulation. 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.

3.4 Activity-dependent redistribution of dNSF1 results in a drastic decrease in its mobility

As described in the preceding section, activity-dependent redistribution of dNSF1^{ST17}-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 dNSF1^{ST17}-EGFP (in a *comi*^{ST17} 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 subjected to photobleaching 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 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 (Fig. 4 B). The mobility of wild-type dNSF1-EGFP was not affected by stimulation (black traces). In contrast, dNSF1^{ST17}-EGFP exhibited a drastic reduction in mobility after the stimulus train (gray traces). These studies reveal that dNSF1^{ST17}-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 dNSF1^{ST17}-EGFP exhibited cytosolic distributions before stimulation, dNSF1^{ST17}-EGFP had a significantly lower mobility (Fig. 4B left). This difference may be explained by enhanced binding between dNSF1 and other synaptic proteins, possibly dSNAP as suggested by the *in vivo* FRET analysis presented in a later section.

3.5 Activity-dependent redistribution SNAP

The preceding findings stimulated efforts to identify 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 SNAPs in promoting binding of NSF to SNAREs (Clary et al., 1990; Weidman et al., 1989), synaptic distribution of a *Drosophila* α - β -type SNAP (dSNAP) (Babcock et al., 2004; Ordway et al., 1994) 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. However, increasing the stimulation frequency to 80 Hz produced a punctate EGFP-dSNAP

pattern and persisted long after the stimulation train (Fig. 5A lower panels) as described for redistribution of dNSF1 in *comatose*. At wild-type synapses maintained at 33 °C, 1 minute of 80 Hz stimulation did not affect the distribution of EGFP-dSNAP (Fig. 5A upper panels). Activity-dependent redistribution of EGFP-dSNAP was quantified as described for dNSF1 (Fig. 5B). 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 (Fig. 5C). 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 as observed for redistribution of dNSF1 (not shown). The activity-dependent redistribution of dNSF1 in *comatose* at 33 °C was similar in response to 20 Hz or 80 Hz stimulation.

Activity-dependent redistribution of the t-SNARE proteins, SYX and SNAP-25, to the PAZ was first observed at adult DLM neuromuscular synapses of *comatose* and revealed a role for NSF in maintaining active zone t-SNAREs during synaptic activity (Kawasaki and Ordway, 2009). At larval neuromuscular synapses, t-SNAREs are not highly restricted to AZs as observed in the adult preparation but rather broadly distributed over the plasma membrane. Nonetheless, larval neuromuscular synapses of *comt^{ST17}* exhibited a consistent accumulation of t-SNAREs at the PAZ after 1 minute of 80 Hz stimulation at 33 °C (Fig. 6). As observed for dSNAP, 1 minute of 20 Hz stimulation under the same conditions did not elicit redistribution of t-SNAREs (not shown). Therefore, activity-dependent redistribution of t-SNAREs and dSNAP in *comatose* may reflect their accumulation in PAZ complexes at the higher stimulation frequency.

3.6 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 applied to both resting and stimulated (80 Hz for 1 minute) boutons at 33 °C as described for dNSF1. Exemplary images are shown in Figure 7A. As expected, 80 Hz stimulation elicited redistribution of dSNAP into a stable punctate pattern. However, EGFP-dSNAP puncta exhibited clear recovery after photobleaching (Fig. 7B) in contrast to the observed immobilization of dNSF1 under similar conditions. While a mild reduction in the mobility of EGFP-dSNAP was observed following stimulation (Fig. 7B), this may reflect enhanced binding of dSNAP and dNSF1 under the restrictive conditions 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 appears to exclude dSNAP as a PAZ anchor for immobilization of dNSF1. Furthermore, the requirement for 80 Hz stimulation to elicit redistribution of dSNAP and t-SNAREs to the PAZ, in contrast to clear dNSF1 redistribution and immobilization observed at 20 Hz, suggests that dNSF1 binding partners beyond the 20S particle participate in immobilization of dNSF1 at the PAZ.

3.7 In vivo interaction between dNSF1 and dSNAP 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; Erickson et al., 2003) 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)

(Shaner et al., 2004). 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, which are in close proximity within the 20S particle (Barnard et al., 1997; Hanson et al., 1997; Hohl et al., 1998; Nagiec et al., 1995; 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-dNSF1^{ST17}) and the C-terminus of dSNAP (dSNAP-tdTomato).

EGFP-dNSF1 or EGFP-dNSF1^{ST17} 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. The resulting images 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). Note that an *FR* >1 indicates FRET whereas an *FR* of 1 is expected in the absence of FRET (Erickson et al., 2001; Erickson et al., 2003). First, FRET was measured at resting synapses (Figure 8A). At 33 °C, clear FRET was detected at resting *comatose* synapses. In contrast, no FRET was detected in *comatose* at 20 °C or in wild type at either temperature. The preceding findings indicate that dNSF1 and dSNAP exhibit increased binding interactions at restrictive temperature in *comatose* even in the absence of synaptic stimulation and redistribution of dNSF1 and dSNAP to the PAZ. This interaction may contribute to the modest reduction in dNSF1^{ST17}-EGFP observed in *comatose* at 33 °C prior to stimulation (Figs. 4 and 7).

Finally, FRET studies were extended to conditions producing activity-dependent redistribution of dNSF1 and dSNAP to examine whether increased interaction was observed as would be predicted for accumulation of NSF-SNAP complexes and 20S particles at the PAZ. Presynaptic boutons were imaged over a five minute period at 33 °C prior to, during and after an 80 Hz stimulation train. Stimulation was applied during the second minute, and FRET was measured at 1, 2, 2.5, 3, 4 and 5 minute time points (Fig. 8). Mean *FR*s were determined for individual time points and plotted over time. As shown in Figure 8B, stimulation of wild-type synapses produced no change in FRET as indicated by *FR*s 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) (Fig. 8B). In contrast, no significant change in FRET was observed at *comatose* synapses in the absence of synaptic stimulation. Rather, *FR*s fluctuated around 1.2 (n=12), as expected from FRET studies of resting synapses (Fig. 8A). For stimulated synapses, comparisons of *FR*s at the 2, 2.5, 3, 4 and 5 minute time points with *FR*s 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.

The above observations demonstrate that acute disruption of dNSF1 activity at *comatose* synapses promotes 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 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.

4. 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, 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} assembles into 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 maps to the D1 domain thought to function in complex disassembly and may not affect SNAP/SNARE binding interactions mediated by the N domain (Nagiec et al., 1995; Whiteheart et al., 1994).

This study also shows that 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 any redistribution of dNSF1 to the PAZ would not be evident in this preparation. Nevertheless, studies in both preparations implicate the PAZ as the site of NSF-mediated SNARE complex disassembly and possible accumulation of 20S complexes following disruption of NSF activity.

In addition, the FRAP and FRET studies reported here indicate that 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. Given that dSNAP exhibits a stable punctate distribution under these conditions but is still mobile and can disassociate from the PAZ, dSNAP may bind to and dissociate from dNSF1 and SNAREs independent of SNARE complex disassembly. Moreover, increased dSNAP binding at the PAZ may reflect accumulation of dNSF1 and t-SNAREs at the PAZ. Unlike dSNAP, dNSF1 is fixed at the PAZ following its redistribution in *comatose*. This observation suggests that dNSF1 is anchored or immobilized at the PAZ by synaptic components which themselves are fixed. For this reason and because dNSF1 is immobilized at the PAZ in the apparent absence of dSNAP or t-SNARE redistribution, synaptic components other than SNAP and SNAREs appear to anchor dNSF1 at the PAZ. These findings suggest that one classical role for SNAP as a Soluble NSF Attachment Protein may not be the primary mechanism for membrane association of NSF in neurotransmitter release.

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 benefit 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 mobilities there. Moreover, 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 insertion complexes at PAZ regions of the presynaptic plasma membrane.

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Figure 1.

Co-immunoprecipitation of endogenous core complexes containing dNSF1, dSNAP and SNAREs. WT and comatose flies were exposed to 38 °C for 10 minutes before decapitation and preparation of head homogenates. A monoclonal SYX antibody (mAb 8C3) was used to pull down endogenous SYX 1A from head homogenates. As a negative control, an equivalent amount of pre-immune mouse IgG was added instead of mAb 8C3. Pre-IP inputs (see materials and methods) were examined to demonstrate an equal amount of target protein in each sample before the IP procedure. Pre-IP inputs and IP samples were blotted for SYX 1A, NSYB, SNAP-25, dNSF1 and dSNAP. **A**, Specific IP of SYX 1A from WT and *comatose* samples. **B**, Co-IP of NSYB, SNAP-25, dNSF1 and dSNAP. Note the increased levels of co-IP from the *comatose* sample. For SYX IPs, ratios of Co-IP for each protein in *comatose* versus wild-type after exposure to 38 °C were: NSYB [1.23 (n=2)]; SNAP-25 [1.39 (n=2)]; NSF [2.20 (n=2)]; and SNAP [1.55 (n=1)].

Figure 2.

Endogenous dNSF1 localization at larval neuromuscular synapses. **A**, Confocal immunofluorescence of dNSF1 at WT synapses relative to the neuronal plasma membrane marker (HRP) and a marker for synaptic vesicles [CYSTEINE STRING PROTEIN (CSP)]. **B**, **C**, Redistribution of endogenous dNSF1 to the PAZ in *comatose* at 33 °C following 1 minute of 20 Hz stimulation (stim). The distribution of dNSF1 was examined with respect to HRP and CSP (**B**) or the AZ marker, BRP (**C**). All images represent maximum projections of 2 optical sections.

Figure 3.

Activity-dependent redistribution of dNSF1 in *comatose*. **A**, Live imaging of wild-type dNSF1-EGFP in a wild-type background or dNSF1^{ST17}-EGFP in a *comt*^{ST17} background. Each presynaptic bouton was first imaged at 20 °C (a) and then exposed to 33 °C and imaged at the indicated time points (b–e). 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.

Figure 4.

FRAP analysis of dNSF1 mobility. **A**, A time series of images of stimulated synapses from WT (dNSF1-EGFP) and *comatose* (dNSF1^{ST17}-EGFP) preparations at 33 °C illustrates photobleaching and recovery. Note the punctate distribution of dNSF1^{ST17}-EGFP prior to photobleaching as well as the absence of recovery. Scale bar = 1 μ m. **B**, FRAP (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. At wild-type synapses, fluorescence recovery (black traces) was not affected by stimulation indicating no change in the mobility of dNSF1-EGFP. In contrast, fluorescence recovery at *comatose* mutant synapses (gray traces) decreased drastically after stimulation indicating a much lower mobility of dNSF1^{ST17}-EGFP.

Figure 5.

Activity-dependent redistribution of dSNAP in *comatose*. **A**, Live imaging of EGFP-dSNAP in a wild-type or *comt*^{ST17} genetic background. Each presynaptic bouton was first imaged at 20 °C (a) and then exposed to 33 °C and imaged at the indicated time points (b-e). 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 ± 0.75 (n=6) and 19.1 ± 2.19 (n=6), which were significantly different.

Figure 6.

PAZ accumulation of t-SNAREs in comatose. Distributions of endogenous SNAP-25 (a–e) and SYX 1A (f–j) were examined by confocal immunofluorescence relative to markers for the presynaptic plasma membrane (HRP; a–j) and the AZ [BRP (a–e) and CAC-tdTomato (f–j)]. comt^{ST17} boutons were maintained at 33 °C and subjected to 1 minute of 80 Hz stimulation prior to fixation. Note the punctate distribution patterns and PAZ localization of SNAP-25 and SYX 1A. All images represent maximum projections of 2 optical sections.

Figure 7. FRAP analysis of SNAP mobility in comatose. *A*, A time series of images of EGFP-dSNAP at stimulated *comatose* synapses at 33 °C illustrates photobleaching and recovery. Note the punctate distribution of EGFP-dSNAP prior to photobleaching and recovery of fluorescent puncta (circles in the 30" image). Scale bar = 1 μ m. *B*, FRAP (as a percentage of maximal recovery) was plotted as a function of time. Each trace represents the mean of 7 independent experiments. Fluorescence recovery within 1 minute decreased from 100% before stimulation (black trace) to approximately 80% after stimulation (gray trace).

Figure 8.

Activity-dependent interactions of dNSF1 and dSNAP revealed through FRET analysis at living synapses. Three-Cube FRET images were obtained from presynaptic boutons co-expressing dSNAP-tdTomato and EGFP-dNSF1 (wild-type background) or EGFP-dNSF1^{ST17} (*comatose* mutant background). **A**, FRET at resting synapses maintained at 20 or 33 °C. For wild type, the mean FRs were 1.04 ± 0.01 at 20 °C (n=17) and 1.02 ± 0.01 at 33 °C (n=19) indicating no FRET. A similar mean FR was observed in *comatose* at 20 °C [1.02 ± 0.01 (n=16)]. In contrast, resting *comatose* synapses at 33 °C exhibited an increased FR of 1.17 ± 0.01 (n=32) which was significantly different from the other three resting conditions. **B**, An activity-dependent increase in FRET was observed at *comatose* synapses (black circles) during 1 minute of 80 Hz stimulation (black bar above the x axis). The mean FRs at 3, 4 and 5 min were significantly higher than the initial mean FR at 1 min (n=13). Stimulated wild-type synapses (open circles) and resting *comatose* synapses (grey circle) exhibited no significant activity-dependent change in FRET.