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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.

Introduction

Previous studies identified core components of the neurotransmitter release apparatus including: N-Ethylmaleimide 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 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.

Results

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, 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

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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 min (Fig. 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).

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 Fig. 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.

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 min 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 min of 20 Hz stimulation had little impact on the distribution of dNSF1–EGFP, which remained similar to that of the 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

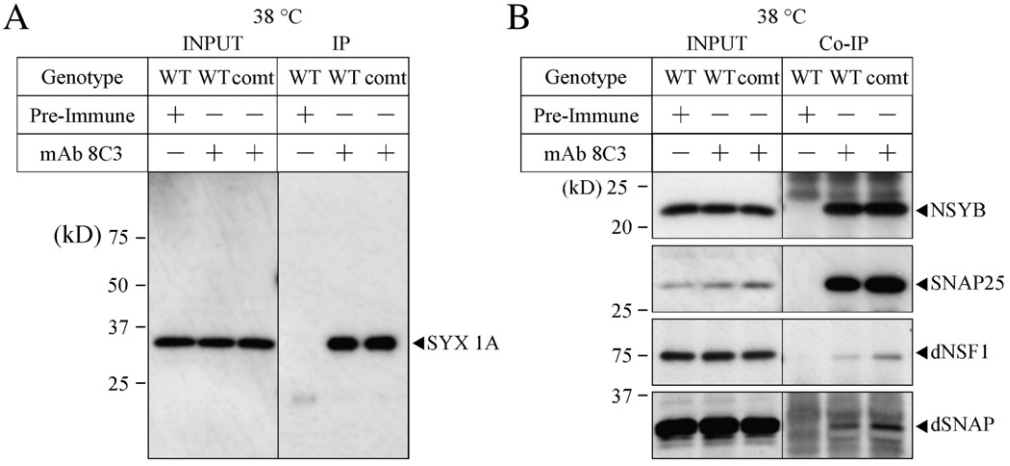

Fig. 1. Co-immunoprecipitation of endogenous core complexes containing dNSF1, dSNAP and SNAREs. WT and comatose flies were exposed to 38 °C for 10 min before decapitation and preparation of head homogenates. A monoclonal SYX antibody (mAb 8C3) was used to pull down endogenous SYX 1A from head homogenates. As a negative control, an equivalent amount of pre-immune mouse IgG was added instead of mAb 8C3. Pre-IP inputs (see Experimental 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 ($n = 1$)].