

Analysis of the mutant *Drosophila* *N*-ethylmaleimide sensitive fusion-1 protein in comatose reveals molecular correlates of the behavioural paralysis

Mahmood Mohtashami,^{*,†} Bryan A. Stewart,[‡] Gabrielle L. Boulianne^{†,‡,§} and William S. Trimble^{*,¶}

^{*}Programme in Cell Biology, [‡]Developmental Biology, Hospital for Sick Children, Toronto, Ontario, Canada

[†]Departments of Zoology, [§]Molecular and Medical Genetics, and [¶]Biochemistry, University of Toronto, Toronto, Ontario, Canada

Abstract

NEM-sensitive fusion protein (NSF) is an ATPase required for many intracellular membrane trafficking steps. Recent studies have suggested that NSF alters the conformation of the SNAP receptors (SNAREs) to permit their interaction, or to uncouple them after they interact. Most organisms have a single NSF gene product but *Drosophila* express two highly related isoforms, dNSF-1 and dNSF-2. dNSF-1 is encoded by the gene *comatose* (*comt*), first identified as the locus of a temperature-sensitive paralytic mutation. Here we show that dNSF-1 is most abundant in the nervous system and can be detected in larval and adult CNS. Subcellular fractionation revealed that dNSF-1 was enriched in a vesicle fraction along with the synaptic vesicle protein synaptotagmin. *comt* flies maintained at the non-permissive temperature rapidly

accumulate sodium dodecyl sulfate (SDS)-resistant SNARE complexes at the restrictive temperature, with concomitant translocation of dNSF-1 from cytosol and membrane fractions into a Triton X-100 insoluble fraction. The long recovery of *comt* flies after heat shock induced paralysis correlated with the irreversibility of this translocation. Interestingly, while dNSF-1 also translocates in *comt*^{TP7} larvae, there is no associated neurophysiological phenotype at the neuromuscular junction (nmj) or accumulation of SDS-resistant complexes in the CNS. Together, these results suggest that dNSF-1 is required for adult neuronal function, but that in the larval nmj function may be maintained by other isoforms.

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The release of neurotransmitter at the synapse occurs via the fusion of a synaptic vesicle with the presynaptic membrane and represents a specialized, and rapid, example of events used throughout the cell to mediate membrane protein trafficking. Insights into this process have been greatly advanced through the study of the mechanisms of membrane fusion, both *in vitro* and *in vivo*. A central player in the regulation of membrane fusion is the ATPase *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF). NSF was first identified as a cytosolic factor required to restore transport activity in an *in vitro* assay of Golgi traffic (Block *et al.* 1988). Subsequent studies have revealed that it is also required for a number of intracellular trafficking steps including endoplasmic reticulum-to-Golgi transport (Beckers *et al.* 1990), epithelial cell transcytosis (Sztul *et al.* 1993), endosome–endosome fusion (Diaz *et al.* 1989; Rodriguez *et al.* 1994; Colombo *et al.* 1996) and neurotransmitter release (Pallanck *et al.* 1995; Schweizer

et al. 1998). Cloning of cDNAs for NSF (Wilson *et al.* 1989) revealed that it contained two ATPase domains highly homologous to domains in a large group of proteins of diverse function, collectively classified as AAA ATPases (for ATPases associated with diverse activities) (Confalonieri and Duguet 1995). Of these two ATPase domains, named D1

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Address correspondence and reprint requests to William S. Trimble, Programme in Cell Biology, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

E-mail: wtrimble@sickkids.on.ca

Abbreviations used: AAA, ATPases associated with diverse activities; ECL, enhanced chemiluminescence; NSF, *N*-ethylmaleimide sensitive fusion protein; PCR, polymerase chain reaction; PNS, postnuclear supernatant; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

and D2, the D2 domain has low ATPase activity (Whiteheart *et al.* 1994) and is responsible for homohexamer formation (Hanson *et al.* 1997) while the D1 domain has highly inducible ATPase activity required for NSF transport function (Whiteheart *et al.* 1994). Interestingly, several other members of the AAA ATPase family are also hexamers with two ATPase domains and are involved in aspects of membrane traffic (Patel and Latterich 1998). However, the majority of intracellular membrane trafficking steps studied to date require NSF.

It is generally thought that NSF acts to establish fusion-competence of membranes by acting on a set of proteins called SNAREs. SNAREs are the receptors of SNAP (Sollner *et al.* 1993b), an adapter molecule that permits NSF to associate with membranes (Clary *et al.* 1990). Some SNARE proteins, including those from *Drosophila*, form stable, SDS-resistant complexes (Hayashi *et al.* 1994; Pevsner *et al.* 1994; Tolar and Pallanck 1998) whose formation is thought to be linked to membrane fusion (Weber *et al.* 1998). NSF can dissociate these stable complexes through ATP hydrolysis (Sollner *et al.* 1993a). Current versions of the SNARE hypothesis of membrane fusion predict that the role of NSF may be two-fold (Hay and Scheller 1997). First, NSF could prime vesicles by uncoupling prefusion SNARE complexes existing in *cis* on membranes to permit their interaction with other SNAREs in *trans* on opposing membranes. This *trans* interaction would be unstable and could proceed to fusion, possibly caused by the release of energy during the progression of complex formation (Chen *et al.* 1999). Second, upon membrane fusion these complexes would reside in *cis* and it is likely that NSF may also act to uncouple these postfusion SNARE complexes to permit their recycling and subsequent reuse.

To date only one isoform of NSF has been found in most species, including yeast and *Caenorhabditis elegans* the complete genomes of which have been sequenced, whereas *Drosophila* possess two highly related NSF isoforms (Ordway *et al.* 1994; Boulianne and Trimble 1995). *dNSF-1* is the gene product of *comatose* (Pallanck *et al.* 1995), and *comatose* (*comt*) mutations have been identified that can cause temperature-sensitive paralysis (Siddiqi and Benzer 1976). While other *Drosophila* synaptic temperature sensitive mutants such as the sodium channel mutation *para*, temperature sensitive alleles of syntaxin, and even the temperature sensitive dynamin mutation *shibire* each display nearly instant paralytic effects on locomotion, *comt* mutations take several minutes to become paralyzed and recovery can take many hours (Siddiqi and Benzer 1976; Littleton *et al.* 1998). We therefore set out to determine the nature of the *comt* mutations and the biochemical properties of dNSF-1.

Previous studies of dNSF-1 have been influential in defining the function of NSF. However, due to the lack of isoform-specific antibodies, those studies have analyzed

dNSF-1 function and the effects of the *comt* mutants, indirectly through changes in SNARE complex abundance. Here we have developed an antibody specific for dNSF-1 to directly examine its distribution and subcellular localization in wild-type and *comt* mutants. We find that dNSF-1 is expressed predominantly in the nervous systems of larval and adult flies, but is only required in the adult.

Experimental procedures

Recombinant protein production

dNSF-2 cDNA (Boulianne and Trimble 1995) was inserted in frame into the pQE30 prokaryotic expression vector (Qiagen, Mississauga, Ontario, Canada) by first PCR-amplifying the 5' end and ligating it into the vector. An internal restriction site was then used to append the remainder of the coding sequence. A dNSF-1 cDNA clone was obtained from the Berkeley *Drosophila* Genome Project (clone no. HL02139), PCR amplified and inserted into the pET-32c expression vector (Novagen, Madison, WI, USA). Both cDNAs were sequenced to ensure PCR fidelity. Recombinant protein was purified using Ni-NTA agarose (Qiagen). The dNSF-1 fusion protein was cleaved from the thioredoxin fusion protein by treatment with thrombin (Sigma, St Louis, MO, USA).

Fly strains and transgenics

Fly stocks were grown using standard *Drosophila* culturing methods. *comt*^{TP7} is a temperature sensitive allele of *dNSF-1* that was generated by O. Siddiqi and kindly provided by K. Krishnan (Tata Institute, Bombay, India). *Oregon-R* was used as the wild-type strain.

Antibody production and western analysis

Anti-dNSF-1 antibodies were generated against a peptide (dNSF-1 amino acids 680–696) conjugated to the activated Keyhole Limpet Hemocyanin carrier protein, Imject (Pierce, Rockford, IL, USA), and injected into rabbits for immunization. This peptide sequence is specific to dNSF-1. The anti-dNSF-1 antibodies were affinity purified using peptides bound to SulfoLink (Pierce). K. Zinsmaier (University of Pennsylvania School of Medicine, Philadelphia, PA, USA) kindly provided us with the cysteine string protein (csp) antibody. Anti-dSyx1A (monoclonal 8C3) was the kind gift of Dr S. Benzer (Cal. Tech., Pasadena, CA, USA). H. Bellen (Baylor College of Medicine, Houston, TX, USA), kindly provided anti-synaptotagmin 2 antibody (dtagmin). Rabbit polyclonal anti- α SNAP antibodies were generated against recombinant protein. HRP-conjugated secondary antibodies were obtained from BIO-RAD (Mississauga, Ontario, Canada). Protein concentrations were determined using the BCA assay (Pierce). Western blots were developed using enhanced chemiluminescence (Amersham, Baie d'Urfe, Quebec, Canada) and exposed to Kodak X-Omat Blue film (Kodak, Rochester, NY, USA). All western blots presented were representative examples of blots from three or more independent experiments. Western blots were scanned and for each band the same area was measured and the data were transferred to GRAPHPAD PRIZM (GraphPad Software Inc., San Diego, CA, USA) for statistical calculations of average and standard error of mean. Statistical significance was determined to be $p > 0.05$ by the one-sample *t*-test.

Tissue preparation for immunocytochemistry

Third instar larvae were dissected in HL3 physiological solution (Stewart *et al.* 1994). The CNS and the muscles including the neuromuscular junctions (NMJ) were left intact and were fixed in 4% paraformaldehyde. Adult head sections were prepared by first dissecting out the proboscis and the air sacs in HL3 physiological solution. The heads were then fixed in 4% paraformaldehyde and placed in 25% sucrose overnight. To prepare them for sectioning, the heads were placed in OCT (Fischer, Nepean, Ontario, Canada) and flash-frozen in 2-methylbutane (Sigma) submerged in N₂ (liquid). The sections were cryosectioned at 12 µm thickness at -20°C. All preparations were permeabilized and blocked in phosphate-buffered saline, pH 7.0 (PBS) plus 5% normal goat serum and 0.1% Triton X-100 before appropriate antibodies were applied. In peptide blocking experiments, 10 ng/mL of the peptide was pre-incubated with the antibody for 30 min before its use in immunocytochemistry. Secondary antibodies used were Alexa 488-conjugated anti-rabbit (Molecular Probes, Eugene, OR, USA) and Cy-3-conjugated anti-mouse (Jackson Immunological Laboratories, West Grove, PA, USA). Tissue preparations were visualized using Zeiss (Heidelberg, Germany) LSM-510 system with the inverted Axiovert 100 M and the images were acquired using the LSM software.

Fly homogenization, subcellular fractionation and SDS-resistant complexes

Fly heads were homogenized and fractionated according to Schulze *et al.* (Schulze *et al.* 1995). Briefly, flies were flash-frozen in liquid N₂ and vortexed to sever the head from the thorax. The body parts were then separated from each other using standard sieves (USA series #25 and #50). The heads were then crushed with mortar and pestle under liquid N₂ and homogenized using a motorized 2 mL Teflon homogenizer in 0.2 M sucrose, 20 mM HEPES buffer, pH 7.4, plus protease inhibitors. The homogenate was centrifuged for 10 min at 1000 g and the supernatant collected as a post nuclear supernatant (PNS). The PNS was then centrifuged at 12 000 g (10 000 r.p.m., JA20 Beckman) for 10 min and the supernatant (S2) collected, diluted 1 : 3 with ice-cold 20 mM HEPES-KOH, pH 7.5, 0.1 M NaCl and layered on top of a sucrose step gradient (10 mL of 0.4 M sucrose, 20 mM HEPES pH 7.5 in the bottom layer, 10 mL of 0.2 M sucrose, 20 mM HEPES, pH 7.5 middle layer). Samples were spun for 2 h at 87 000 g in a swing bucket rotor (SW28 Sorvall). The supernatant at the top layer was collected as the cytosol. The crude synaptic vesicle fraction was withdrawn by a syringe from the 0.2 M and 0.4 M sucrose interface. The larger membrane fragments and cytoskeletal components (P3) were pelleted to the bottom of the tube. To monitor the presence of SDS-resistant complexes before or after the 37°C incubation, the flies were flash-frozen in liquid N₂, then directly resuspended in buffer containing 1% SDS. Since the SDS-resistant SNARE complexes cannot form in SDS, this procedure would permit the observation of only those complexes that had formed during the heat shock. Lysates prepared in this way were electrophoresed and blotted with antibodies specific to syntaxin.

RT-PCR and sequencing

Fly heads of *com1*^{TP7} were crushed to powder under liquid N₂ using a mortar and pestle. Twenty milligrams of the fly head material was weighed and homogenized using the QIA Shredder (Qiagen). Poly

mRNA isolation was done with Oligotex (Qiagen) and reverse-transcribed by AMV.RT (Life Technologies, Burlington, Ontario, Canada) and Superscript KT II (Stratagene, La Jolla, CA, USA) according to the manufacturer's specifications. Samples from the reverse transcriptase reaction were used to perform PCR using oligonucleotides flanking the entire coding sequence. PCR products of the correct molecular weight from two different reactions were collected and further PCR amplified. Those products were then inserted into pBluescript SK⁺ vector (Stratagene, La Jolla, CA, USA) in both orientations and sequenced.

Heat shock and fly collection

For paralysis assays, 10 male flies were tested in each trial (1 trial = 1 *n*). The flies were placed in a prewarmed glass vial that was immersed in a 37°C water bath. In order to analyze the formation of SDS-resistant complexes in the heat shock treated flies, heads of 40 male flies were collected as described above and homogenized directly in PBS, 1% SDS and 1 mM EDTA. SDS-solubilized supernatant was collected after the homogenate was centrifuged at 200 000 g (75 000 r.p.m.) in a TL100.3 rotor (Beckman, Palo Alto, CA, USA). For the fractionation of fly heads into cytosol, Triton X-100 soluble, and SDS-soluble fractions, fly heads were homogenized in ice-cold PBS or HKA (10 mM HEPES, pH 7.5, 140 mM KOAc, 1 mM MgCl₂, 0.1 mM EGTA) containing protease inhibitors and centrifuged at 200 000 g in TL100.3. The supernatant was collected as the cytosol and the pellet was resuspended in PBS containing 2% Triton X-100 and protease inhibitors and incubated on ice for 1 h. The samples were centrifuged again and the supernatant was collected as the Triton X-100 soluble fraction. The pellet was then resuspended in PBS with 1% SDS and 1 mM EDTA, incubated at room temperature (22°C) for 15 min before centrifugation in the same manner. The final supernatant was collected as the SDS-soluble fraction and the SDS-resistant pellet was discarded. For NSF translocation studies, the proportion of NSF in each fraction was calculated as the product of the band density and total protein yield for each fraction.

Electrophysiological recordings

Electrophysiological procedures were carried out as described (Stewart *et al.* 1994; Stewart *et al.* 1996). Maximal excitatory junctional potentials (EJPs) were recorded from larval muscle 6 of segments 3 and 4. HL3 physiological saline contained 1.5 or 2 mM Ca. Bath temperatures were controlled with a Peltier device and controller from Medical Systems Corp (Greenvale, NY, USA). For high temperature recordings larvae were dissected at room temperature and then placed directly into a prewarmed 37°C chamber and allowed to equilibrate for at least 3 min prior to recording.

Results

Drosophila NSF-1 is predominantly expressed in the larval and adult nervous systems

To examine the temporal and spatial distribution of dNSF-1 we raised a specific antiserum against a peptide of sequence unique to dNSF-1. dNSF-1 and dNSF-2 share 84.5% sequence identity so the peptide had to be selected from

non-conserved portions of the protein. The antiserum was then affinity purified on columns of resins to which the peptides had been covalently immobilized. As shown in Fig. 1(a), affinity purified anti-dNSF-1 antibody recognized a single band of 81 kDa in adult *Drosophila* extracts (lane 1) and bound to recombinant dNSF-1 (lane 2, 10 ng, lane 3, 50 ng) but not dNSF-2 (lane 4, 50 ng). Based on the intensity of the signal obtained in 20 µg of total adult fly lysate, we estimate that dNSF-1 represents less than 0.025% of the total protein. The recombinant forms have a slightly reduced mobility on the gels due to the presence of the S purification tag at the N-terminus of the protein contributed by the expression vectors. No cross reactivity to 50 ng of dNSF-2 was seen with the anti-dNSF-1 antibody, even after long exposures. In addition, pre-immune serum did not recognize any bands in the extracts (data not shown).

The antibodies were then used to examine the expression of dNSF-1 during development. As shown in Fig. 1(b), dNSF-1 expression was undetectable in embryonic stages but increased during development. When specific larval tissues were isolated and examined, expression of dNSF-1, was found to be enriched in the isolated larval CNS but not in other secretory tissues such as the salivary gland or in the imaginal discs. The low levels of dNSF-1 seen in the total larval and pupal homogenates (Fig. 1b) was likely contributed by the CNS where expression is relatively high

(Fig. 1c). In adults, expression of dNSF-1 also appeared to be enriched in the head (Fig. 1c, head lane) compared with the rest of the body (Fig. 1c, body lane), further suggesting that dNSF-1 may be primarily expressed in neurons.

Immunofluorescence microscopy confirmed that dNSF-1 expression is indeed restricted to the CNS. As shown in Fig. 1(d), isolated larval CNS discs revealed strong expression within the neuropil, where staining significantly overlapped with that of cysteine string protein (Fig. 1e). Overlapping expression is seen in yellow in the merged image (Fig. 1f). dNSF-1 was also detected in the larval neuromuscular junction (Fig. 1g) and the stained boutons can be seen to overlay the muscle cells in the merged fluorescence/DIC image (Fig. 1h). This neuromuscular staining was specific and could be blocked by pre-incubation of the antibody with the immunizing peptide (Fig. 1i). Frozen sections of adult heads were stained with anti-dNSF-1 and 22C10, an antibody that detects postmitotic neurons in the peripheral nervous system, and, respectively, detected with Alexa-488 and Cy3-conjugated secondary antibodies. These results demonstrate that dNSF-1 is abundantly expressed within the three main optic neuropil layers which include the lamina, medulla, and lobula complexes (lobula and lobula plate). In contrast, very little expression is observed within retinal neurons (Figs 1j and k).

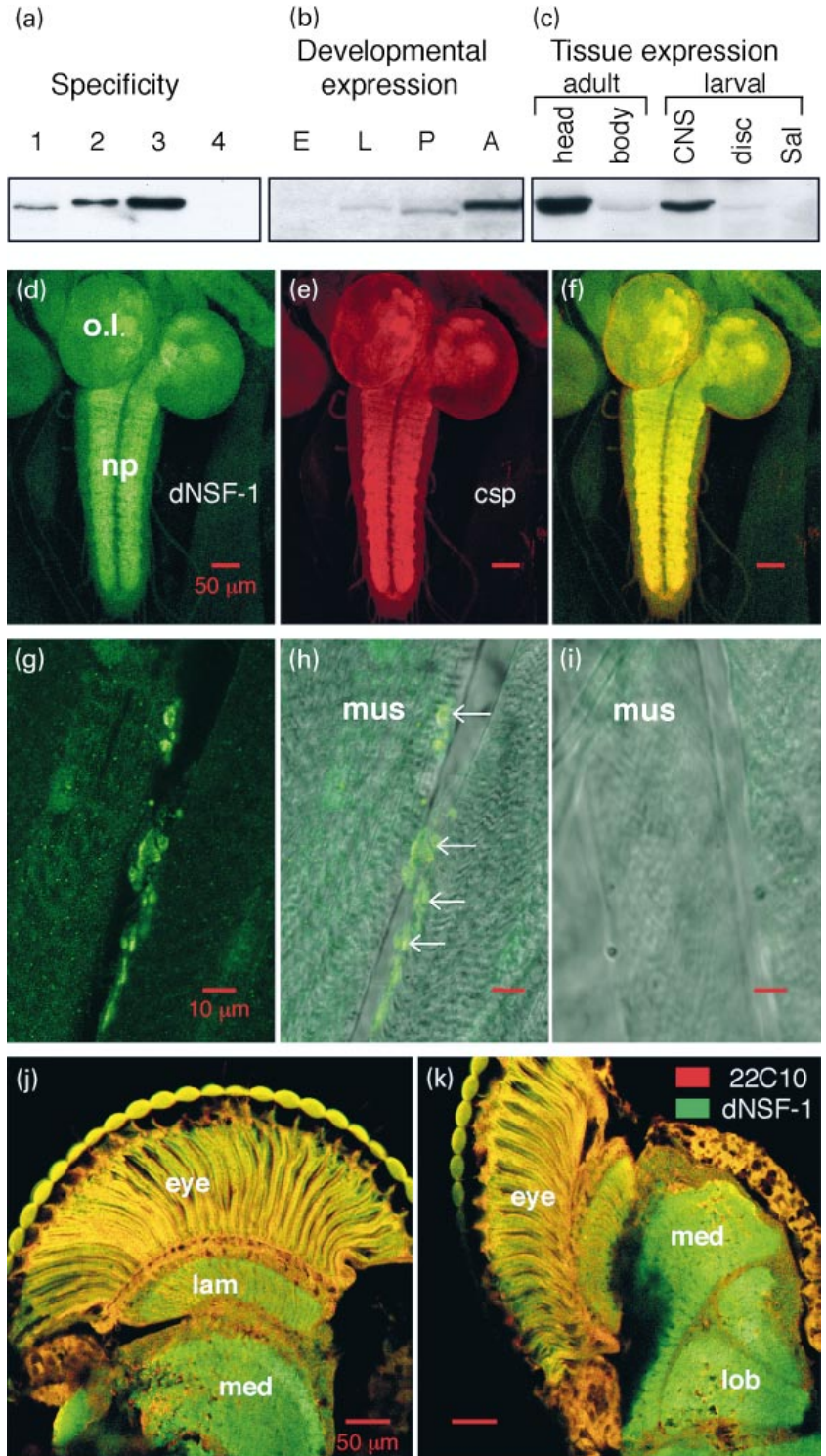
Fig. 1 dNSF-1 expression during development. (a) dNSF-1 antibody identified a single band in the adult fly lysate (lane 1) containing 20 µg of total protein. Antibodies to dNSF-1 recognized 10 ng and 50 ng of purified recombinant 6XHis-dNSF-1 fusion protein (lanes 2 and 3, respectively) but not 50 ng of purified 6XHis-dNSF-2 protein (lane 4). (b) Twenty micrograms of TCA-precipitated protein lysate from *Drosophila* at different developmental stages were probed with the anti-dNSF-1 antibody (E, embryo 0–24 h.; L, third instar larvae; P, white pupae; A, adult Oregon R flies). dNSF-1 expression progressively increases during development with the highest level at the adult stage. Note that dNSF-1 at the pupal stage is detected as a band having lower molecular weight than that of the adult and larval stage. This may be due to degradation, post-translational modification or the result of alternative splicing. (c) Twenty micrograms of extracts of adult head, body, and larval CNS, imaginal discs (disc) and salivary glands (Sal), were probed with anti-dNSF-1 antibody. Despite the low levels of dNSF-1 protein in the third instar larvae, abundant dNSF-1 expression was observed in the larval CNS but not salivary glands or imaginal discs. Also, the adult fly head seems to contribute much of the dNSF-1 protein detected in the whole adult lysate since the level of dNSF-1 expression detected in the adult body is very low. Similar results were obtained for 3 other experiments. (d, e and f). Immunofluorescence microscopy of larval CNS. Third instar larval CNS were dissected and probed for dNSF-1 and cysteine string protein (csp). Confocal images demonstrate that dNSF-1 expression (d, green) is present throughout the CNS, particularly enriched within the neuropil (np) of the ventral ganglia where synapses occur. The detection of the synaptic protein,

cysteine-string protein (csp), also in the neuropil of the CNS (e, red) corroborates the presence of dNSF-1 at the synapses. The merger of staining (f, yellow) shows that the neuropil is the strongest site of overlap. In other sites of CNS such as in the optic lobes (ol) and the ventral ganglia cortex immunoreactivity to dNSF-1 is at very low levels but that of csp is absent. (g, h and i). Neuromuscular junctions (NMJ) staining with anti-dNSF-1 is inhibited by peptide. (g) and (h) show the different images of the same preparation of NMJ. Whereas in (g) the dNSF-1 staining highlights a typical synapse at the NMJ, in (h) the differential interference contrast (DIC) image allows muscle (mus) visualization as well as the dNSF-1 staining of the synapses (arrows). Similar to H, the image in I contains both the DIC and the fluorescent image. However, NMJ staining in I is inhibited in the presence of the peptide (10 ng/mL) that the antibody was originally raised against. Other peptide competition studies that were done on larval CNS staining and on western blots confirmed the inhibition of antibody by the peptide (data not shown). (j and k) Immunofluorescence microscopy of adult head sections. Adult heads were flash frozen and cryosectioned prior to immunostaining with antibodies against 22C10 which predominantly labels postmitotic neurons within the peripheral nervous system and dNSF-1. Similar to several other independent head sections, confocal microscopy of the two sections presented here reveal that the green dNSF-1 staining predominated in the three major optic neuropils including the lamina (lam), the medulla (med) and the lobula complex (lob). In contrast, only low levels of dNSF-1 were observed in photoreceptor neurons in the eye which are abundantly labelled with 22C10 (red).

dNSF-1 is abundant in the synaptic vesicle fraction

We used an adult head preparation to examine the biochemical properties of dNSF-1 through the fractionation scheme depicted in Fig. 2(a). As can be seen in Fig. 2(b), dNSF-1, was present in all fractions with the highest

concentrations being found in the crude synaptic vesicle fraction. Vesicle fractionation was demonstrated with antibodies to synaptotagmin (dtagmin) which was greatly enriched in the vesicle fraction. In contrast, a control for plasma membrane fractionation, the plasma membrane



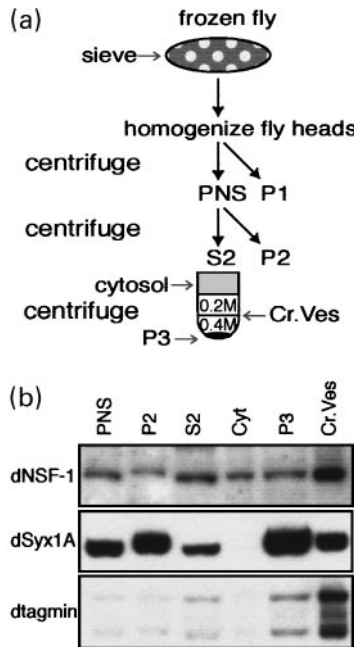


Fig. 2 dNSF-1 is enriched in the crude synaptic vesicle fractions. (a) Wild-type Oregon-R fly heads were homogenized and fractionated according to the depiction in this figure and as described in the Materials and methods (Schulze *et al.* 1995). Post-nuclear supernatant (PNS) was fractionated into P2 and S2 through centrifugation. The supernatant S2 fraction was then placed on a step gradient containing 0.2 M and 0.4 M sucrose and centrifuged. The resultant pellet containing plasma membranes and cytoskeleton was designated P3 fraction; the material at the 0.2 M and 0.4 M sucrose interface was the fraction containing the crude vesicles (Cr.Ves). The cytosol (Cyt, light gray) did not enter the 0.2 M sucrose buffer. (b) Blots containing 10 μ g of total protein from each fraction were probed with anti-dNSF-1, dSyx1A and dSynaptotagmin (dtagmin) antibodies indicated at the left. dtagmin antibody recognized full-length synaptotagmin (upper band) and a degradation product (lower band) in all fractions tested. This figure is representative of three independent experiments.

tSNARE protein Syntaxin (dSyx1A) was shown to be abundant in the P3 fraction and absent from the cytosolic fraction. The lower levels of syntaxin in the crude vesicle fraction may either reflect contamination of this fraction with membranes, or may represent syntaxin in synaptic vesicles since previous studies have shown that a significant amount of syntaxin is found in vesicles. These results further support the possibility that dNSF-1 may have a function important in synaptic vesicle release.

Biochemical analysis of *comt*^{TP7}

To investigate the function of dNSF-1 in more detail, we first characterized an existing mutation within the *dNSF-1* locus, the *comatose* (*comt*). Several alleles of *comt* have been shown to be the result of point mutations within the ATPase domain (Pallanck *et al.* 1995) and such mutations

can be informative about structurally important residues within the protein. We therefore decided to identify the mutation responsible for a particularly potent allele of *comatose*, *comt*^{TP7}. Two independent cDNAs of dNSF-1 from *comt*^{TP7} were obtained by RT-PCR and sequenced to completion. Only one mutation was observed in both clones, a substitution of serine for proline at amino acid 398, identical to the results reported by others (Tolar and Pallanck 1998; Kawasaki and Ordway 1999).

To determine the biochemical correlates of paralysis seen when *comt*^{TP7} flies were shifted to the non-permissive temperature, homogenates were prepared from the heads of flies maintained for various times at 37°C and analyzed for the presence of SDS-resistant SNARE complexes containing dSyx1A. Similar to previous reports (Littleton *et al.* 1998; Tolar and Pallanck 1998), very few high molecular weight complexes containing dSyx1A exist in wild-type Oregon R flies at any temperature, while SDS-resistant complexes rapidly accumulate in *comt*^{TP7} flies after shift to 37°C (Fig. 3a). When the samples are boiled and probed for dSyx1A, complexes break up and the monomeric dSyx1A protein is seen at equal amounts (Fig. 3a, lower panel).

Redistribution of dNSF-1 accompanies accumulation of SDS-resistant complexes on *comt*^{TP7}

The location of the mutation in dNSF-1 within a conserved domain of NSF, together with the rapid paralysis and the accumulation of SDS-resistant SNARE complexes in *comt*^{TP7} flies shifted to the non-permissive temperature, raised the question of what happens to the dNSF-1 protein during this heat shock paradigm. We observed that even at the permissive temperature (HS 0 min), *comt*^{TP7} flies contain less dNSF-1 and more SDS-resistant complexes than do OreR flies, raising the possibility that the temperature sensitivity of dNSF-1 could result from protein degradation. However, the levels of dNSF-1 protein before and after heat shock treatment in total lysates from OreR or *comt*^{TP7} flies do not appear to dramatically change (Fig. 3a, middle panel) making acute degradation of dNSF-1 an unlikely explanation for the paralysis. Since the overall levels of dNSF-1 protein did not change enough to explain the rapid paralysis during heat shock, we investigated whether its fractionation properties were altered. We therefore probed fractions of wild-type or *comt*^{TP7} flies held for various periods of time at 37°C for dNSF-1. In these experiments flies were incubated for the times indicated, then flash frozen to permit removal and homogenization of their heads in HKA buffer. The homogenate was then spun at 200 000 g to collect the supernatant that represents the cytosol. As shown in Fig. 3(b), similar to that observed in total fly extracts, the cytosol of *comt*^{TP7} flies contains significantly less dNSF-1 than do Ore-R flies, even at the permissive temperature (compare *comt*^{TP7} cytosol HS 0 min to Ore-R cytosol, HS 0 min). Within 2 min at 37°C,

reductions in the levels of dNSF-1 were seen from the cytosolic fraction of *comt^{TP7}* flies while no change was seen in the Oregon R flies over this time. Similarly, no change was seen in the distribution of α SNAP in these flies. 100% paralysis of *comt^{TP7}* homozygotes was typically seen within 3 min (data not shown).

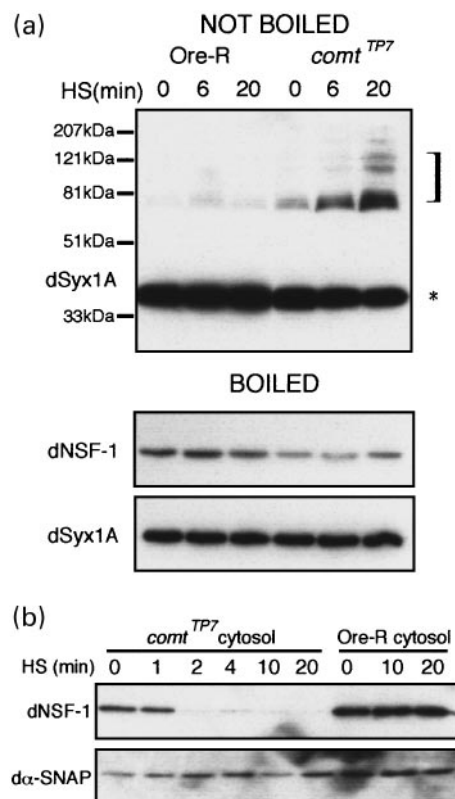
Since there was a large reduction in the dNSF-1 levels in the cytosol (Fig. 3b), we then investigated if the dNSF-1 protein was moving to another fraction. For this, cytosolic fractions were prepared as above, then the pellet was resuspended with buffer containing 2% Triton X-100, incubated on ice for 1 h, then spun again for 30 min at 200 000 g. The supernatant was taken as the Triton X-100 soluble fraction and the pellet was then resuspended in buffer containing 1% SDS, incubated and spun again. The SDS soluble supernatant fraction was kept and the SDS-insoluble pellet was discarded. As can be seen in Fig. 4(a), dNSF-1 disappeared concomitantly from the cytosol soluble fractions while appearing to accumulate in the SDS-soluble fraction. Quantification of the results of four experiments, shown in Fig. 4(b), reveals that a portion of the protein lost

from the cytosol and Triton X-100 soluble fractions appears to have accumulated in the SDS soluble fraction. No change in the fractionation properties of dNSF-1 is seen in Oregon R flies upon heat shock treatment. The translocation of dNSF-1 from soluble to insoluble fractions correlated with loss of NSF activity and may represent a denatured or precipitated form of the protein. After heat shock the *comt^{TP7}* flies remained immobile for prolonged periods after they were brought back to permissive temperature (data not shown). There was no return of the dNSF-1 protein back to the cytosolic fraction after a 30-min recovery period at room temperature (Fig. 4c). Similar redistribution of dNSF-1 protein was obtained with another allele of *comatose*, *comt^{ST17}* (data not shown).

Redistribution of dNSF-1 in *comt^{TP7}* larvae has no physiological consequences

Our observation that dNSF-1 was expressed in both the adult and larval CNS raised the possibility that *comt^{TP7}* larva may be affected by loss of dNSF-1 at the non-permissive temperature. However, initial experiments revealed that even after prolonged exposure to 37°C, larvae remained mobile (data not shown). To determine if the dNSF-1 protein expressed in larvae underwent the temperature-dependent translocation seen in adults, we examined the effect of heat shock on the presence of dNSF-1 in cytosol prepared from the larval CNS. Indeed, as Fig. 5a shows, at

Fig. 3 Incubation of *comt^{TP7}* flies at non-permissive temperatures result in SDS-resistant SNARE proteins accumulation and loss of cytosolic dNSF-1 despite no change in total dNSF-1 protein. (a) SDS-resistant SNARE complexes rapidly accumulate in *comt^{TP7}* at the non-permissive temperature. Wild-type Oregon-R (Ore-R) and *comatose* flies were flash-frozen in liquid N₂ immediately after shift to 37°C for 0, 6 or 20 min. The heads were collected directly in SDS-containing buffer and homogenized. Half of the amount of each sample was boiled and the other half was not in order to preserve SNARE complexes. Ten micrograms of each sample was loaded per lane and detected by anti-dSyx1A antibody. No paralytic effect of 37°C was seen on wild-type Ore-R flies, nor did SDS-resistant complexes accumulate in this fly line. In contrast, shift of *comatose* mutants to 37°C resulted in a time-dependent increase in SDS-resistant SNARE complexes at MW 70 kDa to 200 kDa (J) as seen in the upper panel. There was also a corresponding decrease in the monomeric form of dSyx1A (*) at MW 37 kDa. The lower panel shows that there are no differences in the amount of monomeric dSyx1A once the samples are boiled (exposure time on film equal to 1/6 the exposure of the figure in upper panel). Heat shock treatment of the *comt^{TP7}* flies did not result in any change in the total dNSF-1 protein levels as seen in the middle panel (similar results were obtained in three independent experiments). (b) dNSF-1 levels rapidly decline in the cytosol upon heat shock. After 37°C treatment for different periods of time (0–10 min), *comt^{TP7}* fly heads were obtained as before, homogenized in non-detergent containing PBS buffer, and centrifuged to obtain the cytosol. The immunoreactivity of dNSF-1 protein in the cytosol rapidly decreased upon prolongation of 37°C treatment, while the cytosolic d- α SNAP protein detection remained unchanged in the same flies. There was no change in immunodetection of either dNSF-1 or d- α SNAP upon shift of Ore-R flies to 37°C. (20 μ g of protein was loaded per lane, $n = 3$).



the non-permissive temperature there was a depletion of the cytosolic component of dNSF-1 whereas there was no change in the level of cytosolic α -SNAP. This observation is similar to that seen in the adult head, suggesting that in larvae the protein is also sensitive to temperature. We then wanted to determine if this translocation also resulted in the accumulation of SDS-resistant SNARE complexes as occur in the adult head. There were more 7S SNARE complexes in CNS tissue from larvae *comt*^{TP7} than in OreR at both temperatures. Surprisingly, however, as shown in Fig. 5(b), larvae maintained at the non-permissive temperature for more than 10 min showed no increased accumulation of SDS-resistant complexes above controls that were not HS treated. In fact, in both *comt*^{TP7} and Ore-R there appeared to be fewer complexes following shift to 37°C in each of three independent experiments. The significance of this decrease

is not known, but since this is the opposite of that seen in adults, it further suggests that dNSF-1 activity is not required for complex disassembly in the larval CNS.

To more directly determine if dNSF-1 contributes to the physiological properties of the larval nervous system, excitatory junction potentials were measured at the neuromuscular junction of third instar larvae following direct stimulation of the motor axon. As shown in Fig. 5(c), stimulation at a frequency of 1 Hz resulted in potentials that were equivalent at recording temperatures of 22°C and 37°C. Moreover, repeated stimulation gave superimposable traces lacking the rundown characteristic of the *comt* phenotype in

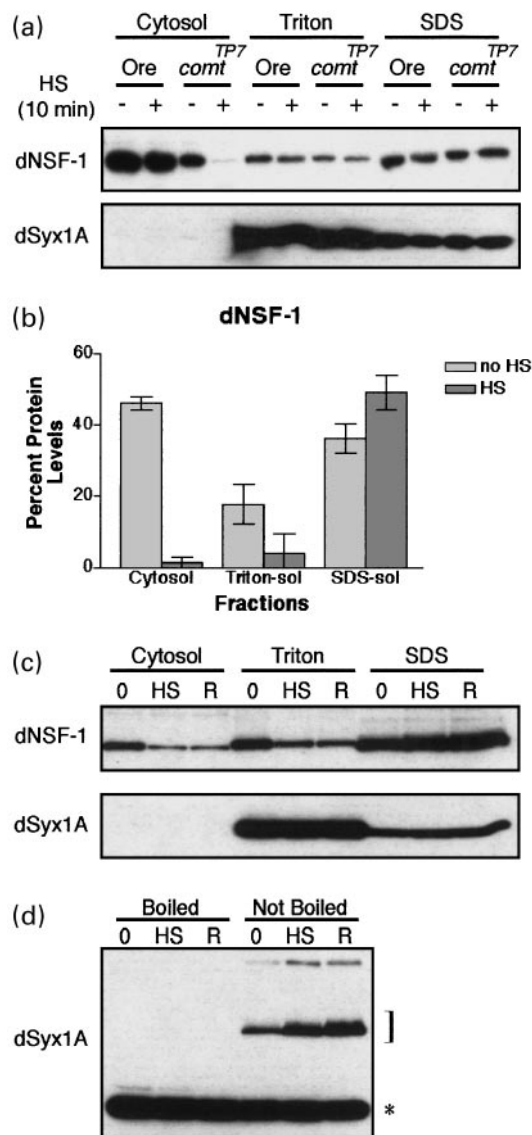


Fig. 4 Heat shock (HS) of *comt*^{TP7} induces dNSF-1 irreversible translocation from cytosolic to SDS-soluble fraction. (a) dNSF-1 translocates from the cytosol to insoluble fractions. HS-treated (+) and room temperature (-) OreR and *comt*^{TP7} fly heads were fractionated into cytosol, 2% Triton X-100 soluble and 1% SDS-soluble fractions. 20 μ g of each fraction was probed with anti-dNSF-1. In the OreR flies, there is no change in the distribution of dNSF-1 protein between HS treated and those placed at room temperature. However, in the *comt*^{TP7} flies, dNSF-1 protein disappears from the cytosol upon shift to 37°C, but its immunoreactivity is increased in the SDS-soluble fraction. There is no difference in distribution of the SNARE protein, dSyx1A after incubation at room temperature or 37°C despite an increase in SDS-resistant complex formation. (b) Histogram shows the average of four experiments for the *comt*^{TP7} flies as in (a). The bands detected by anti-dNSF-1 in each lane were measured by densitometry and presented as the percent of the total dNSF-1 detected in all three fractions. HS treatment resulted in a significant decrease of dNSF-1 protein in the cytosolic ($p < 0.01$) and Triton X-100 soluble ($p < 0.05$) fractions. In contrast, it resulted in a significant increase in the SDS-soluble fraction ($p < 0.05$). Error bars indicate standard error. (c) Thirty minutes incubation at permissive temperature after HS does not result in relocalization of dNSF-1 protein to cytosol in *comt*^{TP7} flies. *comt*^{TP7} flies were allowed to recover for 30 min at room temperature after a 10 minute HS period. However, all flies remained immobile at the bottom of chamber after this treatment. Heads of the treated flies (R) were homogenized and fractionated as in (a) and compared with the flies that were not treated (0) or just placed at non-permissive temperature for 10 min (HS). dNSF-1 translocation from cytosolic and Triton X-100 soluble fractions to SDS-soluble fraction is similar to that indicated in (b). However, 30 min of recovery period after HS did not result in any change in the amount of dNSF-1 protein in any of the fractions. (d) Accumulation of SDS-resistant SNARE complexes is not effected by 30 min of recovery period in *comt*^{TP7} flies. Flies were treated to a recovery period as in (c). Samples of total homogenates of fly heads were prepared the same way as in 3a), and were probed for SDS-resistant SNARE complexes using anti-dSyx1A antibody. Half the samples were boiled to demonstrate equal loading at the monomeric level (*) and the other half were not boiled to preserve SNARE complexes (]). When compared with the non-treated samples (0) there was an accumulation of SDS-resistant complexes in the 37°C HS treated samples (HS) which did not diminish after a 30-min period recovery (R) at room temperature.

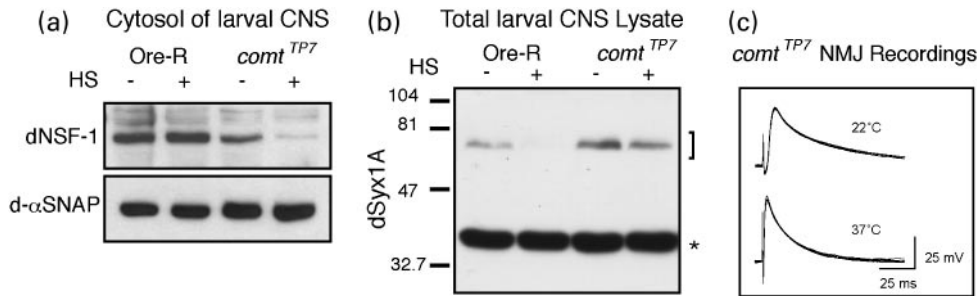


Fig. 5 Redistribution of dNSF-1 in *comt*^{TP7} larvae. (a) dNSF-1 disappears from the larval CNS cytosol during heat shock. *comt*^{TP7} larvae were placed in permissive (–, room temperature) and non-permissive (+, 37°C) temperatures for 10–15 min then dissected and the CNS homogenized in PBS. As a negative control, heat shock treated Oregon-R (Ore-R) larval CNS was included. Ten micrograms of CNS cytosol was loaded in each lane, probed with anti-dNSF-1 antibody. The blot was probed with anti-d-αSNAP antibody to demonstrate equal loading. Similar results were obtained in 2 other independent experiments. (b) Heat shock does not lead to the accumulation of SDS-resistant complexes. To determine the amount of SDS-resistant complexes formed in permissive and non-permissive temperatures, total larval CNS lysates from above were placed in SDS containing buffer immediately after dissection and electrophoresed without boiling. Fifteen micrograms of each sample was loaded per lane. Blots were probed with anti-dSyx1A. The position of molecular weight markers is indicated at the left. Monomeric

dSyx1A (*) migrated at MW 37 kDa and SDS-resistant SNARE complexes as detected by anti-dSyx1A antibody migrated around MW 70 kDa (J). Similar results were obtained in 2 other independent experiments. (c) Paralysis does not occur in the neuromuscular junction of third instar *comt*^{TP7}. Suprathreshold EJPs were recorded at 22°C and 37°C in 1.5 mM extracellular calcium. There was no apparent difference in EJP amplitude or response to repetitive stimulation at high temperature. Similar results were obtained in four separate experiments and also with OreR flies as controls at both temperatures (data not shown). The traces show every third response in a 30 pulse train delivered at 1 Hz. The apparent difference in fall time between the traces is a reproducible, temperature-dependent change seen in both *comt*^{TP7} and OreR flies (data not shown). Similar stimulation paradigms elicit obvious stimulation-dependent reduction in synaptic transmission in *comt* adult NMJs (Kawasaki *et al.* 1998).

the adult neuromuscular junction (Kawasaki *et al.* 1998). These results indicate that despite its expression in the larval CNS, dNSF-1 does not appear to contribute a necessary function for neurotransmitter release at the NMJ and it is not necessary to inhibit the accumulation of SDS-resistant complexes.

Discussion

While several previous biochemical and physiological studies have used the *comt* alleles to support the general role of dNSF-1 in SNARE complex breakdown, they lack in direct analysis of the consequence of the *comt* mutations on dNSF-1 function. Using an isoform specific antibody we show, for the first time, that dNSF-1 is expressed abundantly in both the adult and larval CNS. Importantly we also found an abnormal shift in the subcellular distribution of dNSF-1 in *comt* alleles at the restrictive temperature suggesting the main effect of the mutation may be to make the protein unavailable to carry out its normal function. dNSF-1 is abundantly expressed in larval CNS including the presynaptic neuromuscular junction, and we have found that the mutant protein behaves similarly to heat shock in both adults and larvae. Surprisingly, however, *comt*^{TP7} larvae display no overt paralytic or physiological phenotype suggesting the lack of functional importance of dNSF-1 in synaptic transmission in larval stages.

To gain structural insights into important residues within dNSF-1, we sequenced the *comt*^{TP7} allele and found that it results from a proline to serine substitution at amino acid 398 within the D1 ATPase domain. This residue is conserved in all of the NSF proteins identified to date and is also conserved in all known members of the *Saccharomyces cerevisiae* AAA ATPase family (numbered residue 211 as described in Fig. 1b of the review by Patel and Latterich (1998). In a survey of other known AAA ATPases in Genbank, very few had substitutions at this residue. Notably, however, orthologs of the PEX6 peroxisome biosynthesis gene product consistently had alterations at this site. In humans and *Schizosaccharomyces pombe* (fission yeast), the residue is an asparagine while, more intriguingly, a serine is found in this position in PEX6 from rat, *Pichia pastoris* and *Yarrowia*. The presence of a naturally occurring serine in these latter proteins implies that a serine can substitute functionally at this position, as it does in dNSF-1 at the permissive temperature. However, other modifications to PEX6 in these species may provide the protein with thermostability not seen in the dNSF-1 mutant. This residue is also conserved within the D2 domain of NSFs for which the structure has been determined (Lenzen *et al.* 1998). The D2 protomer consists of an ATP-binding domain and a helical domain and, according to this structure, the proline residue would be found adjacent to the loop that connects these two domains. If the same general

structure holds for the D1 domain, its position near this linker region may have functional consequences for the D1 domain itself or may affect its interactions between D1 and D2. In contrast, mutations in two other alleles of *comatose* were found to be due to widely spaced substitutions within the D1 domain: G274E in *comt^{st17}* and S483L in *comt^{st53}* (Pallanck *et al.* 1995). As both *comt^{TP7}* and *comt^{ST17}* both underwent translocation of the dNSF-1 protein into insoluble fractions following heat shock, it is likely that this translocation accounts for the similar temperature-sensitive paralytic phenotype.

comt^{TP7} flies paralyze within minutes, yet their recovery is very slow and often on the order of hours [Littleton *et al.* (1998) and our data not shown]. Even after recovery periods of more than 30 min the majority of dNSF-1 remains in the SDS-soluble fraction and SDS-resistant SNARE complexes persist (Figs 4c and d). This suggests that the protein inactivation by heat shock may be permanent and recovery may require new protein synthesis. Interestingly, when *comt^{TP7}* flies are incubated for only 2 min in the non-permissive temperature, dNSF-1 rapidly becomes depleted from the cytosolic fraction and increases in the SDS-soluble fraction. We could not account for all of the dNSF-1 following heat shock, suggesting that a portion of the protein was either degraded or remained insoluble following boiling in SDS. The SDS-soluble fraction in this case may represent aggregates of dNSF-1 or protein associated with higher order structures making it no longer available for use. Since recycling of SNARE proteins is likely to be required for continual function of the nervous system, the paralysis of *comt^{TP7}* flies is likely due either to the depletion of functional SNARE proteins through their loss into complexes, or the accumulation of complexes at the release sites blocking further release.

It remains unclear why *Drosophila* needs two highly related isoforms of NSF. It is intuitive that they must have different functions since the adult *comt^{TP7}* flies become paralyzed within minutes at the non-permissive temperature despite the presence of dNSF-2. It is possible that dNSF-1 might be the isoform specialized for functions at the adult nerve terminal. For example, recent studies have revealed that mammalian NSF has additional functions in regulating receptor activities through physical interactions with the GluR2 subunit of the glutamate receptor (Nishimune *et al.* 1998; Osten *et al.* 1998; Song *et al.* 1998) and with the β -arrestin protein (McDonald *et al.* 1999). However, as we and colleagues (Littleton *et al.* 1998; Tolar and Pallanck 1998) have shown, it is clear that at the non-permissive temperature *comt^{TP7}* flies accumulate SDS-resistant SNARE complexes indicating a requirement for dNSF-1 in their dissociation. Lack of SNARE dissociation may be the primary defect in these mutants, but dNSF-1 cannot be solely responsible for this task as we show that it is only weakly expressed in the embryo where other proteins must

carry out this task. Also, even though dNSF-1 expression is detected in the larval CNS, no phenotype has been attributed to its loss in *comt^{TP7}* larvae at the non-permissive temperature. In fact, it is not clear if dNSF-1 expressed in the larval CNS is functional since SDS-resistant complexes do not accumulate in larvae maintained at the non-permissive temperature (Fig. 5b). We presume therefore the dNSF-2 must be responsible for neural activity in embryos and larvae, whereas dNSF-1 functions primarily in adults. The larval neuromuscular junction is the most commonly used preparation to study neurotransmission in *Drosophila*. Therefore it is important to note that dNSF-1 does not have an essential role in larval neurotransmission at this synapse. If dNSF-1 and dNSF-2 represent specialized isoforms of NSF that have diverged to possess essential but non-overlapping roles in neurotransmitter release, it will be important to define their individual roles and determine if other species also utilize this plurality in specific tissues or developmental stages.

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