

FAST TRACK

Dominant-Negative NSF2 Disrupts the Structure and Function of *Drosophila* Neuromuscular Synapses

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ABSTRACT: N-ethylmaleimide sensitive fusion protein (NSF) is an ATPase necessary for vesicle trafficking, including exocytosis. Current models hold that NSF is required in a step that readies vesicles for fusion by disassembling postfusion SNARE protein complexes allowing them to participate in further rounds of vesicle cycling. Whereas most organisms have only one NSF isoform, *Drosophila* has two. dNSF1 is the predominant functional isoform in the adult nervous system. Conditional mutations in the dNSF1 gene, *comatose*, are paralytic and lead to disruption of synaptic transmission and the rapid accumulation of SNARE complexes in adult flies. This isoform is not required for synaptic transmission in larvae. In contrast, dNSF2 is important at earlier developmental stages, and its broad expression indicates its importance in neural and non-neural tissues alike. To study dNSF2, and to circumvent the lethality of

dNSF2 null mutants, we have constructed transgenic flies carrying a dominant negative form of dNSF2. When this construct was expressed in neurons we observed suppression of synaptic transmission, activity-dependent fatigue of transmitter release, and a reduction in the number of releasable vesicles. However, we unexpectedly found that there was no accumulation of SNARE complexes accompanying these physiological phenotypes. Intriguingly, we also found that expression of mutant dNSF2 induced pronounced overgrowth of the neuromuscular junction and some misrouting of axons. These results support the idea that dNSF2 has multiple roles in cellular function and adds that not all of its functions require disassembly of the SNARE complex.

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INTRODUCTION

Neurotransmitter release occurs by the regulated fusion of synaptic vesicles with the synaptic plasma

membrane. This form of secretion has been shown to have many molecular similarities with other membrane transport and secretory pathways. A key molecule that regulates membrane fusion is N-ethylmaleimide sensitive fusion protein (NSF). NSF was initially identified by its requirement in Golgi trafficking (Block et al., 1988), and has been subsequently shown to be important in many membrane fusion events, including synaptic transmission. NSF is an ATPase that has two ATPase domains and is a member of the triple-A ATPase group of proteins. The two ATPase domains of NSF have diverse functions: the D1 domain is required for ATPase-dependent NSF

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function while the D2 domain is required for homo-hexamers formation (Whiteheart et al., 1994).

Through an adaptor protein called α -SNAP (soluble NSF attachment protein) NSF can bind a protein complex known as the SNARE (SNAP receptor) complex (Sollner et al., 1993). This complex, which consists of VAMP, Syntaxin, and SNAP-25, is thought to be the minimal requirement for membrane fusion (Weber et al., 1998). The ATPase activity of NSF is capable of dissociating the SNARE complex (Sollner et al., 1993). The current model of NSF function holds that NSF is required to dissociate postfusion SNARE complexes to permit their reuse (Littleton et al., 2001).

Although most organisms have only one NSF gene, two homologs of NSF have been cloned from *Drosophila*, *dNSF1*, and *dNSF2* (Ordway et al., 1994; Boulianne and Trimble, 1995; Pallanck et al., 1995b). *dNSF1* was found to be the gene product of *comatose*, a temperature sensitive paralytic mutant (Pallanck et al., 1995a). Studies of *comatose* adult flies revealed that SNARE complexes accumulate at the restrictive temperature (Littleton et al., 1998; Tolar and Pallanck, 1998). This correlates with locomotory paralysis, an accumulation of docked vesicles and reduction in neurotransmitter release under repetitive stimulation (Kawasaki et al., 1998). *dNSF2* is likely the functional NSF isoform at earlier *Drosophila* developmental stages (Golby et al., 2001; Mohtashami et al., 2001). Interestingly, Mohtashami et al. (2001) found that, despite the high levels of *dNSF1* in larval CNS, SNARE complexes do not accumulate in *comatose* larvae nor do they show any physiological phenotypes associated with the mutations in *dNSF1*. This suggests that *dNSF2* is the predominant functional NSF isoform at larval synapses.

Drosophila mutants lacking functional *dNSF2* die as embryos or first instar larvae (Golby et al., 2001), likely because *dNSF2* is involved in many cellular events during development. To circumvent this, and to enable studies of synaptic transmission at mature larval synapses, we have used the Gal4-UAS expression system (Brand and Perrimon, 1993) to target dominant negative NSF2 proteins to the presynaptic nerve terminal. Whiteheart and colleagues (Whiteheart et al., 1994) showed that NSF-sensitive transport activity can be eliminated by single amino acid substitutions in the first nucleotide binding region of NSF and that a single mutant molecule within the NSF homo-oligomer can disrupt normal NSF function. Therefore, we have expressed mutant *dNSF2* protein containing amino acid substitutions identical to those described in Whiteheart et al. (1994). Here we show that expression of mutant *dNSF2* affects *Drosophila* larval synaptic development and physiology.

MATERIALS AND METHODS

Fly Stocks

Drosophila were raised on standard medium at 25°C.

The wild-type strain used was *Oregon-R*. The following transgenic strains were used in this study: *P{GawB}elav^{C155}* is a viable Gal4 enhancer trap strain (herein called *elav^{C155}-Gal4*) with the Gal4 containing P-element under the regulatory control of the endogenous *elav* enhancer. The endogenous *elav* gene product is expressed in a pan-neuronal pattern starting early in development (Robinow and White, 1988) (gift of C. Goodman). *P{GAL4-*elav.L*}3A* is a Gal4 strain (herein called *elav^{3A}-Gal4*) with the *elav* promoter fused in frame with *Gal4* sequence. It is a viable insert on the third chromosome (gift of C. Goodman). *shi^{ts1}*, *P{GawB}elav^{C155}* is a recombinant line containing the *elav^{C155}* Gal4 driver and the temperature-sensitive allele *shibire^{ts1}*; a mutation of the gene encoding dynamin that blocks endocytosis at restrictive temperature (Koenig and Ikeda, 1989). For experiments using *shi^{ts1}*, 31°C or greater was used as the restrictive temperature. *P{GAL4-Mhc.W}MHC-82* is a Gal4 strain (herein called *MHC-Gal4*) with the myosin heavy chain promoter fused in frame with *Gal4* sequence. This Gal4 construct is expressed in muscle from late embryos onward (Schuster et al., 1996a,b) (gift of C. Goodman). *P{GawB}how^{24B} 24B-Gal4* is a Gal4 enhancer trap strain (herein called *24B-Gal4*) that expresses in mesoderm early in embryonic development and in all larval muscles (Brand and Perrimon, 1993). *UAS-fasciclinII* is a viable third chromosome UAS strain that expresses Fas II (Schuster et al., 1996a,b) (gift of C. Goodman). *UAS-dNSF2^{WT}* is a UAS strain containing the entire wild-type *dNSF2* open reading frame. *UAS-dNSF2^{E/Q}* is a UAS strain containing the *dNSF2* open reading frame with a PCR constructed glutamate to glutamine amino acid substitution at position 326. The generation of this point mutant and the transgenic fly stocks is described in (Stewart et al., 2001). *elav^{3A}-Gal4: UAS-dNSF2^{E/Q}* is a strain with a recombinant third chromosome containing both the Gal4 and UAS constructs as described above. Homozygous *elav^{3A}-Gal4:UAS-dNSF2^{E/Q}* are lethal, with very few larval and adult escapers, and are thus maintained over the balancer chromosome *TM3, y⁺, Sb*.

Throughout this study males of the appropriate UAS line crossed to virgin females of the appropriate Gal4 line gave rise to larvae expressing mutant or wild-type *dNSF2*. Control animals were therefore the uncrossed UAS or Gal4 lines or the Gal4 line crossed to *UAS-dNSF2^{WT}*. We have generated several independent *UAS-dNSF2^{E/Q}* lines whose phenotypic effects range from early first instar lethality to complete viability when crossed to *elav^{C155}-Gal4*. The physiological phenotypes reported here were confirmed (data not shown) using *UAS-dNSF2^{E/Q}* lines that produce earlier lethality but yield some animals that escape to third instar larvae.

Analysis of SDS-Resistant Complexes

The CNS from 10–20 third instar larvae were dissected in PBS buffer and immediately immersed in PBS plus 1% SDS and homogenized. Twenty micrograms of protein lysate, determined by BCA assay (Pierce, Rockville, IL), was separated by SDS-PAGE. The proteins were then transferred to PVDF membrane and the blots probed for *Drosophila* Syntaxin using mAb 8C3 at a concentration of 1:1000. Preparation of SNARE complexes from adult heads was performed exactly as described in (Mohtashami et al., 2001).

Electrophysiology

Wandering third instar larvae were dissected in HL3 medium as described in Stewart et al. 1994. Calcium was added to the solution at the concentrations indicated in the text. Electrophysiological procedures used are described (Stewart et al., 1994, 1996). Briefly, the two-electrode voltage clamp mode of an Axoclamp 2B amplifier (Axon Instruments Inc., Burlingame CA) was used to measure nerve-evoked and spontaneous synaptic currents. Borosilicate glass pipettes were pulled to have a resistance of approximately 20 M Ω when filled with 3 M KCl. The clamp was tuned to have a settling time of 1 ms or less in response to 20 mV hyperpolarizing voltage steps from the holding potential of -80 mV. For nerve stimulation the cut end of the appropriate segmental nerve was taken into a suction pipette (8–10 μ m diameter) and current was delivered to the nerve via an Isolator-11 (Axon Instruments Inc) stimulus isolation unit. Data were digitized at 10 kHz, acquired and analyzed with Pclamp 8 software (Axon Instruments Inc). Data were sampled from muscle fiber 6 of abdominal segments 3 and 4.

Bath temperature control for experiments using *shibire* mutant alleles was achieved using a Peltier device and temperature controller from Medical Systems Corp. (Greenville, NY).

Immunocytochemistry

Third instar larvae were dissected in HL3 medium or PBS, fixed in 4% formaldehyde for 10–20 min, washed in PBT (PBS plus 0.1% Triton X-100) for 30 min, blocked with 1% normal goat serum in PBT for 30 min, and then incubated in the indicated primary antibody for 2 h at room temperature or 4°C overnight. FITC conjugated goat anti-HRP (Cappel/ICN Biomedical, Costa Mesa, CA) was used at 1:1000; mouse anti-Fasciclin II (mAb 1D4; gift of C. Goodman) was used at 1:5. For immunofluorescent applications larvae were incubated in the appropriate fluorescent secondary antibody for 2 h at room temperature, washed in PBT 30 min for 1 h, mounted and cleared in 70% glycerol containing 2% 1,4-diazabicyclo[2.2.2]octane (DABCO). Samples were imaged and reconstructed for morphological analysis on a Leica confocal microscope. For immunohistochemical applications larvae were incubated in the appropriate biotinylated secondary antibody, washed for 30 min in PBT, incubated with avidin-HRP of the Vectastain kit according

to the manufacturer's directions (Vector Laboratories, Burlingame, CA). Final visualization was achieved by reacting the preparation in 3 μ g/mL diaminobenzidine and 0.03% hydrogen peroxide. The length of NMJ branches was measured using Northern Lights software and a Nikon Optiphot2 microscope.

Electron Microscopy

Third instar *elav^{C155}*, *elav^{C155}; UAS-dNSF2^{WT}*, and *elav^{C155}; dNSF2^{E/Q}* wandering larvae were dissected in HL3 saline. Dissected larvae were processed for electron microscopy as described (Jia et al., 1993), with the addition of 4–9% sucrose to the fixative. Tissue was embedded in EMBED (Electron Microscopy Sciences, Inc), and random sections (75–100-nm thick) of neurons innervating muscle 6 and 7 were collected.

Data Analysis

Data were compiled, analyzed, and mathematical curve fitting was performed with Prism 3 (Graphpad Software, San Diego). Unpaired Student's *t* tests or one-way ANOVA were used to test for statistical differences between the group means with $p < .05$ chosen as the level of significance. Data are presented as mean \pm S.E.M. graphically and throughout the text. Sample sizes (*n*) are the number of cells unless otherwise indicated.

RESULTS

To investigate the function of dNSF2 in *Drosophila* we constructed a point mutation in the ATP binding region of the D1 domain of dNSF2 following the work of Whiteheart et al. (1994). ATP binding domains have a consensus sequence known as the DEAD box. Each nucleotide binding subdomain of dNSF2 contains a modified binding domain: DEID. Substitution of the glutamic acid for a glutamine residue within this domain is predicted to eliminate ATP hydrolysis but not nucleotide binding. Indeed, Whiteheart et al. (1994) demonstrated that this mutation in mammalian NSF eliminates NSF-dependent Golgi transport activity measured in cell-free extracts. We have previously shown that mutation of glutamate 326 of the dNSF2 D1 domain reduces NEM-sensitive ATPase activity of dNSF2 by approximately 50% (Stewart et al., 2001). To express the mutant protein we created transgenic flies carrying UAS-dNSF2^{E/Q} and UAS-dNSF2^{WT} constructs for use in the Gal4-UAS expression system, as previously described (Stewart et al., 2001).

Several independent UAS insertion lines were generated and expressed with the pan-neuronal driver *elav^{C155}-Gal4*, the muscle specific driver *MHC-Gal4*,

and the mesoderm and muscle driver *24B-Gal4*. When expressed with *elav^{C155}-Gal4* some of the UAS lines caused embryonic lethality while others caused lethality during second and third instar larval stages. One of the UAS lines, *dNSF2^{EC2}* was viable when expressed with *elav^{C155}-Gal4*. Expression with *24B-Gal4* similarly caused lethality at different developmental stages depending on the UAS line used, and again *dNSF2^{EC2}* was viable. These data show that in addition to its role in the nervous system dNSF2 likely plays important developmental roles in the muscle and mesoderm. *MHC-Gal4* crossed to *UAS-dNSF2^{EC2}* was also viable, and we examined these larvae for defects in synaptic transmission, including miniature excitatory junctional current amplitude, but found no defects. *24B-Gal4* and *MHC-Gal4* lines were not further examined. We attribute the differing effectiveness of the UAS lines to their sites of insertion. The remainder of the work in this article was done using *UAS-dNSF2^{EC2}* crossed to *elav^{C155}-Gal4* or *elav^{3A}-Gal4*, or with the *elav^{3A}-Gal4:UAS-dNSF2^{E/Q} / +* recombinant strain.

Synaptic Morphology

Because NSF is known to be widely involved in vesicular membrane trafficking it may be involved in developmental processes such as axon extension,

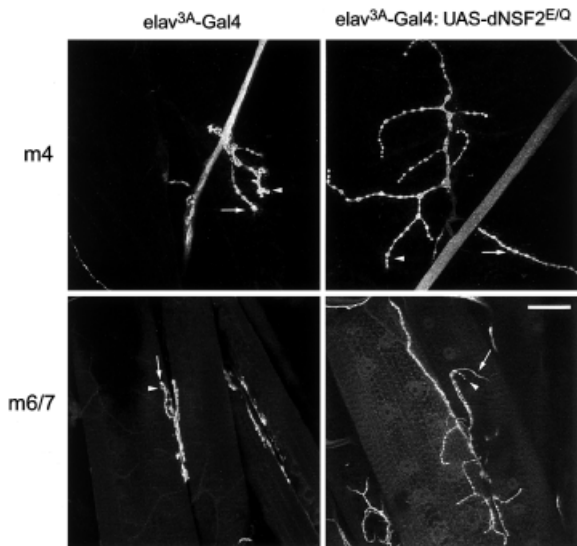


Figure 1 Synaptic overgrowth in *dNSF2^{E/Q}* larvae. Increased branch length and branch numbers were observed in larvae expressing *dNSF2^{E/Q}*. Examples from NMJs on muscle 4 (upper panels) and muscles 6/7 (lower panels) from *elav^{3A}-Gal4* controls and *elav^{3A}-Gal4: UAS-dNSF2^{E/Q}* larvae are shown. The NMJs were labeled with Fluorescein conjugated anti-HRP. Scale bar: upper panels, 15 μm ; lower panels, 40 μm .

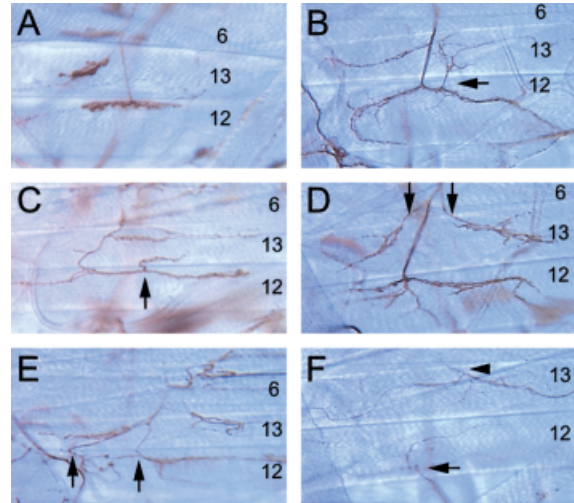


Figure 2 Branching defects in larvae expressing *dNSF2^{E/Q}*. Examples of synaptic morphology from muscle 12/13 from (A) Control and (B–F) mutant larvae. (B,C) The arrows point to abnormal branches that reach back from muscle 12 to muscle 13. (D) The arrow points to multiple nerve entry points onto muscle 13. (E) The arrow points to multiple nerve entry points onto muscle 12. (F) Misrouting of axons under muscle 13; the arrowhead points to muscle 13 innervation while the arrow points to muscle 12 innervation abnormally placed on the lateral side of the muscle.

growth cone movement, and the expansion of the neuromuscular junction. We, therefore, determined if there were any effects on the formation of the NMJ in larvae expressing *dNSF2^{E/Q}* using standard immunocytochemical techniques. Surprisingly, rather than a reduction in NMJ morphology, as might be predicted if *dNSF2^{E/Q}* were generally limiting membrane trafficking, we observed an expansion of the nerve terminal, which was most apparent as a lengthening and elaboration of the branching pattern (Fig. 1). To quantify this change we measured the length of visible branches of the two motor neurons that innervate muscles 6 and 7 in segments 3 and 4. *elav^{3A}-Gal4: UAS-dNSF2^{E/Q} / +* larvae had total branch lengths of $715 \pm 29 \mu\text{m}$ ($n = 6$), while *elav^{3A}* controls had branches $454 \pm 42 \mu\text{m}$ ($n = 6$) ($p < .001$) long. There was no effect of expressing *UAS-dNSF2^{WT}* on synaptic morphology. Thus, the NMJs of larvae expressing the mutant protein were about 60% longer than controls.

In a small fraction ($\sim 10\%$) of the hemisegments examined we also noticed inappropriate branching and misrouting of axons to some NMJs (Fig. 2). This was most apparent for the NMJs of muscle 12 and 13. Normally the axons that innervate these muscles send a single posterior branch to muscle 13 and then pass over the dorsal surface of that muscle to innervate muscle 12 at a single point of innervation. In larvae

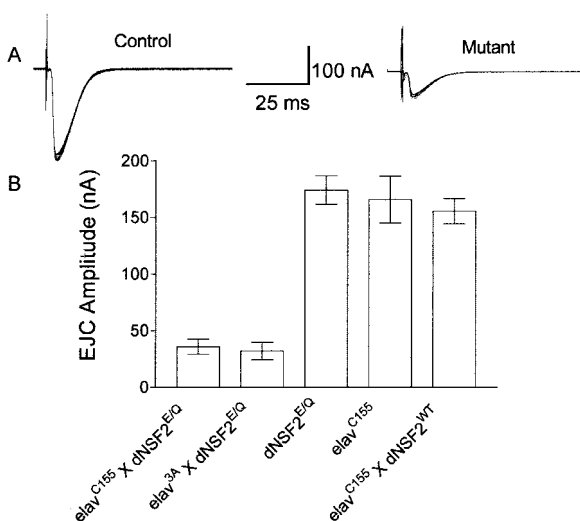


Figure 3 Impaired synaptic transmission in $dNSF2^{E/Q}$ expressing larvae. (A) Examples of synaptic currents recorded from controls and larvae expressing $dNSF2^{E/Q}$. (B) Summary of EJC amplitudes collected from three control strains and from larvae expressing $dNSF2^{E/Q}$ under the control of two different *elav* constructs—*elav*^{C155}, and *elav*^{3A}. These data were collected at 0.1 Hz stimulation frequency in 1.5 mM Ca^{2+} HL3 solution from muscle 6 of abdominal segments 3 and 4.

expressing $dNSF2^{E/Q}$ we observed multiple sites of innervation on both muscle 12 and muscle 13. In addition, axons sometimes “reached back” from muscle 12 to innervate muscle 13. Rarely, we also saw that the axons that innervate muscle 12 passed under muscle 13, rather than over, on their way to innervate muscle 12.

Analysis of Evoked Synaptic Transmission

Expression of $dNSF2^{E/Q}$ in neurons with *elav-Gal4* drivers led to a substantial reduction in nerve-evoked synaptic current measured at larval neuromuscular junctions (Fig. 3). When the motor neurons were stimulated at 0.1 Hz in 1.5 mM extracellular calcium, we measured synaptic currents of 35.9 ± 6.6 nA ($n = 6$) and 34.5 ± 12.4 nA ($n = 6$) in animals expressing $dNSF2^{E/Q}$, whereas the control lines had currents of 174 ± 12.4 nA ($n = 6$) and 165.9 ± 20.0 nA ($n = 6$). When *elav*^{C155} was crossed to $dNSF2^{WT}$, currents of 155.6 ± 11.2 nA ($n = 6$) were observed, indicating that the simple overexpression of NSF protein cannot account for the reduction in evoked synaptic currents in the mutants. The mean amplitudes of the mutant lines are statistically different from the control lines ($p < .001$; ANOVA).

We next measured maximal evoked synaptic cur-

rents in extracellular calcium concentrations ranging from 0.35 to 3.0 mM to determine if the Ca^{2+} dependency of release was affected [Fig. 4(A)]. The control animals exhibited a large nonlinear increase in synaptic current amplitudes with increasing extracellular calcium that reached the maximum amplitude of ~ 200 nA at 2.0 mM calcium. This appeared to be a near saturating level of calcium under these conditions. The mutant animals also showed a calcium dependent increase in transmitter release; however, the maximal attainable current was reduced to a level of approximately 35 nA. We estimated the Ca^{2+} cooperativity of release by fitting linear regression lines to log-transformed data [Fig. 4(B)], as described in Stewart et al. (2000). Interestingly we found that the slope of the line from the $dNSF2^{E/Q}$ larvae ($n = 2.1$) was significantly reduced compared to that of the controls ($n = 3.1$) (ANCOVA, $p < .05$).

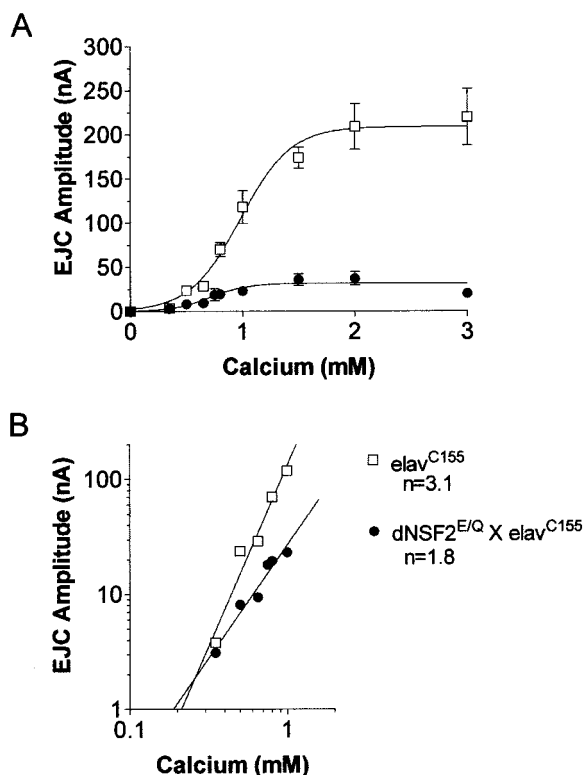


Figure 4 Reduced calcium cooperativity. (A) Evoked junctional currents were recorded from control and mutant larvae in 0.35–3.0 mM Ca^{2+} HL3 solution. Each point represents the mean value obtained from 6–9 cells in the controls and 6–12 cells in the mutants. Where visible, the error bars are S.E.M. (B) Calcium cooperativity (n) was estimated by calculating the slope of linear regression lines to log-transformed data up to 0.8 mM Ca^{2+} for the mutants and up to 1.0 mM Ca^{2+} for the controls. These Ca^{2+} levels represent 50% of the maximum EJC amplitude at saturating Ca^{2+} .

Spontaneous Transmitter Release

Although the reduction in nerve evoked synaptic current was obtained with a neural specific driver, we sought to ensure there was no effect on the sensitivity of the postsynaptic glutamate receptors by analyzing spontaneous miniature synaptic events [Fig. 5(A) and (B)]. We measured the amplitude of approximately 100 mEJCs from each of four different muscles. We obtained mean mEJC amplitudes of 0.51 ± 0.02 nA for the *dNSF2^{E/Q}* line, 0.53 ± 0.01 for *elav^{C155}*, and 0.60 ± 0.01 nA for *dNSF2^{E/Q}* crossed to *elav^{C155}*. These mean values are not statistically different ($p > .26$; ANOVA) and indicate that the postsynaptic neurotransmitter receptors are not altered in the mutant animals and thus cannot account for the reduction in evoked current amplitude.

We did, however, notice a marked reduction in the frequency of mEJCs in mutant animals [Fig. 5(C)]. Neurons expressing *dNSF2^{WT}* had a mEJC frequency of 3.1 ± 0.1 Hz and the two control lines individually had frequencies of 3.3 ± 0.1 Hz and 4.1 ± 0.6 Hz. However, in larvae expressing *dNSF2^{E/Q}* the mEJC frequency was only 0.8 ± 0.1 Hz ($p < .001$; ANOVA). Because the amplitude distribution of the mEJCs is similar in control and mutant lines, this represents a genuine reduction in frequency and not simply a loss of small mEJCs in the background noise in mutant animals.

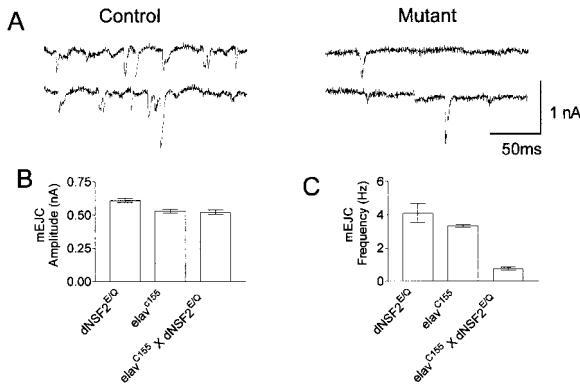


Figure 5 Reduction in mEJC frequency but not amplitude. (A) Traces of mEJCs recorded from control and mutant larvae. (B) The mean amplitude of mEJCs obtained from approximately 400 individual events was not different among the controls and mutants. (C) Summary of mEJC frequency. The average rate of spontaneous release was measured in five to seven cells for each genotype. Larvae expressing *dNSF2^{E/Q}* had significantly reduced mEJC frequency.

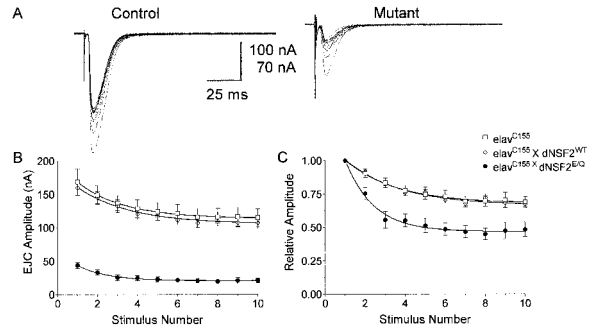


Figure 6 Increased fatigue of synaptic transmission. (A) Ten consecutive traces of synaptic currents obtained at 1 Hz stimulation in 1.5 mM Ca²⁺ obtained from *elav^{C155}* × *dNSF2^{E/Q}* and control larvae. (B) Absolute EJC amplitudes and (C) relative amplitudes obtained from six cells of each genotype. Single exponential decay curves were fit to the data with time constants of 0.4 for controls and 0.7 for the mutants.

Synaptic Fatigue

Previous reports of *dNSF1* function in adult *comatose* flies revealed a decrease in the neurons ability to maintain transmitter release under continuous stimulation. To investigate if expression of *dNSF2^{E/Q}* caused a similar phenotype at larval synapses we stimulated the nerve at moderate frequencies and indeed found enhanced fatigue of neurotransmission. Stimulation of 1.0 Hz in 1.5 mM calcium caused a reduction in synaptic currents during the first few pulses of a stimulus train in both the control and mutant animals. However, the control animals maintain transmission at about 70% of the very first pulse of the train, whereas the mutant animals fatigue to about 45% of the first pulse (Fig. 6). The rate of fatigue is also faster in the mutants. The time constant of exponential curves fit to the data are 0.71 for the mutants and 0.37 and 0.38 for the control lines. Thus, not only do the mutant animals fatigue to a lower plateau level they do so at a faster rate than control animals.

We also found that synaptic fatigue is dependent on the frequency of stimulation and not on the number of stimuli delivered. In animals expressing the mutated NSF synaptic current amplitude plateaus at 90 and 84% of the amplitude of the first pulse at stimulus frequencies of 0.1 and 0.2 Hz, while at 0.5 or 1.0 Hz transmission fatigues to the lower plateau of 45% (data not shown). To be certain that the lower initial output of the mutants was not biasing our fatigue measurements we attempted to equalize the initial output of the mutants and controls by reducing the extracellular calcium concentration to 0.5 mM, a level

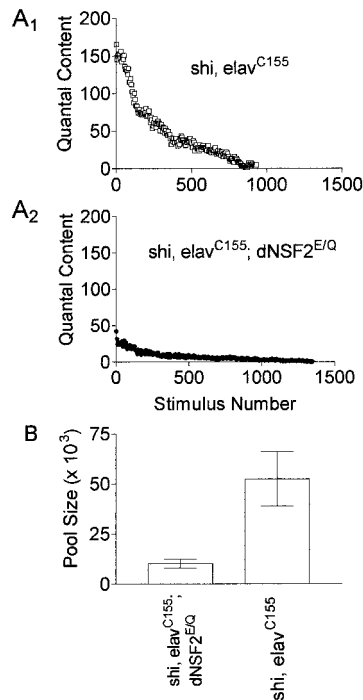


Figure 7 Reduced pool of releasable vesicles. Larvae of the genotype *shi, elav^{C155}* and *shi, elav^{C155}; dNSF2^{E/Q}* were stimulated at 3 Hz at 31°C. (A₁, A₂) Examples from one experiment of the decay in quantal content over the duration of the stimulus trains. For clarity, five consecutive data points were averaged and plotted. (B) The total number of quanta released in each experiment were counted and the mean values from seven mutant and eight control cells are shown.

at which the initial output is more similar, but we found that the mutant animals still fatigue to a lower plateau than controls (data not shown).

Estimates of Vesicle Pool Size

One interpretation of these physiological data on neurons expressing mutant dNSF2 is that there are fewer functional vesicles available to be released. To determine if this were the case, we used the *shibire^{ts1}* mutant allele of dynamin to block endocytosis. By recording synaptic currents, at restrictive temperature (31°C), while stimulating the motor nerve to depletion, we estimated the total pool of releasable vesicles (Fig. 7). To carry out this experiment we used a recombinant *shi^{ts1}; elav^{C155}* line crossed to *dNSF2^{E/Q}* to create male larvae carrying *shi^{ts1}* and expressing *dNSF2^{E/Q}*. We found that the recombinant *shi^{ts1}, elav^{C155}* controls had $52,400 \pm 13,500$ ($n = 8$) releasable vesicles while the *shi^{ts1}, elav^{C155}; dNSF2^{E/Q}* line had only $10,200 \pm 2,200$ ($n = 7$) releasable vesicles,

which is a statistically significant difference ($p < .01$). Our estimate for the wild-type vesicle pool size is similar to that recently reported by (Delgado et al., 2000). This result shows that neurons expressing the mutant dNSF2 have fewer releasable vesicles.

Because there was a clear reduction in the pool size we examined the synaptic ultrastructure of larvae expressing *dNSF2^{E/Q}* by electron microscopy (Fig. 8). We observed an abundance of small clear synaptic vesicles within the nerve terminal in both controls and larvae expressing the mutant NSF. Thus, there does not appear to be a major defect in synaptic vesicle biogenesis or transport within the nerve terminal.

No Effect on SNARE Complex Abundance

Previous studies of the *comatose* mutation in adult *Drosophila* revealed that SNARE complexes accumu-

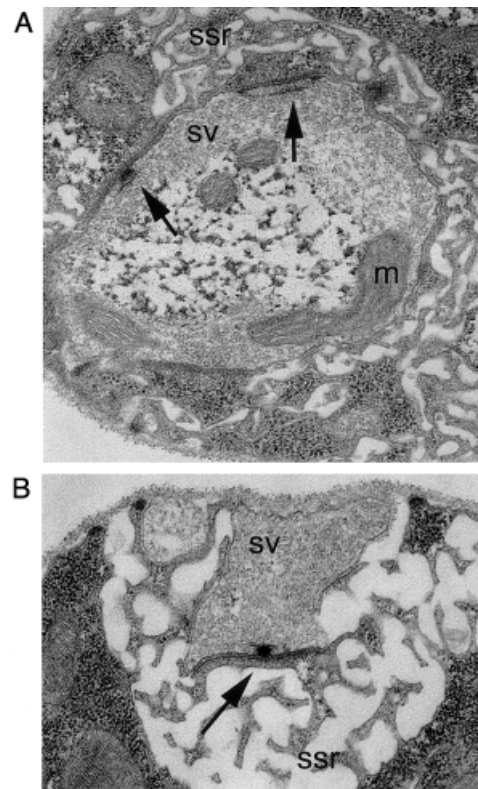


Figure 8 Synaptic ultrastructure. (A) *elav^{C155}* and (B) *elav^{C155}, dNSF2^{E/Q}* NMJ synapses both show active zone densities and an abundance of small, clear synaptic vesicles. The mutant terminals were somewhat smaller and narrower than the controls. These images are representative of random samples taken from three larvae of each genotype. ssr, subsynaptic reticulum; sv, synaptic vesicle; m, mitochondria. Arrows point to active zone T-bars.

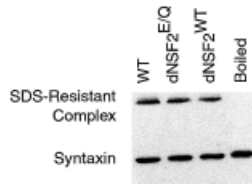


Figure 9 No accumulation of SNARE complexes. Western blots of protein prepared from adult heads were probed with anti-Syntaxin antibodies. The antibody detects monomeric Syntaxin (~30 kDa) as well as SDS-resistant SNARE complexes at ~70 kDa. The complexes are completely dissolved by boiling. There was no detectable change in the level of SNARE complexes in either $dNSF2^{E/Q}$ or $dNSF2^{WT}$ expressing flies. The result is representative of five independent experiments.

late rapidly when dNSF1 function is impaired. This is taken as evidence that the ATPase activity of the molecule is necessary to break down the complex and maintain a supply of SNARE monomers. Because we know that the $dNSF2^{E/Q}$ mutant impairs the ATPase activity of the molecule, we sought to determine if accumulation of SNARE complexes correlates with the phenotypes reported here. We, therefore, analyzed protein extracts from adult heads and third instar larval brains by Western blot. To our surprise we could not find any evidence for accumulation of SNARE complexes (Fig. 9). Similar levels of ~70 kDa SDS-resistant SNARE complexes were detected in all genotypes. This result suggests that the phenotypes we observed in the larvae expressing the mutant protein do not result from the loss of SNARE proteins into complexes.

DISCUSSION

In this article we report on the developmental and physiological effects of expressing a dominant negative form of dNSF2 at *Drosophila* larval NMJs. We have previously shown (Stewart et al., 2001) that the mutation inhibits the ATPase activity of the molecule by 50%, and Whiteheart et al. (1994) have shown that one mutant molecule among the NSF oligomer is sufficient to inhibit the activity of the whole complex. Additionally, we and others (Golby et al., 2001; Mohtashami et al., 2001), have previously presented data to indicate that dNSF2 is the predominant functional NSF isoform at the larval synapse. Because we are expressing the mutant protein in a background that is otherwise wild-type for dNSF2, we presume that some dNSF2 oligomers will not incorporate a mutant molecule, and thus some wild-type dNSF2 activity will remain. This is the likely explanation for the

remaining neural function and the viability of the larvae. Thus, larvae expressing the mutant protein are likely to be similar to hypomorphic alleles of the gene.

In several respects our physiological data are similar to those previously described for adult neurotransmission in *comatose* mutants of dNSF1. We see impaired neurotransmitter release, greater synaptic fatigue, and a reduced supply of releasable vesicles, as has been reported for *comatose* (Kawasaki et al., 1998). In contrast, we did not observe an increase in SNARE complex abundance as seen in *comatose* adults (Littleton et al., 1998; Tolar and Pallanck, 1998), although we expected the $dNSF2^{E/Q}$ mutant to impair SNARE complex disassembly (Nagiec et al., 1995). It remains possible that the remaining dNSF2 ATPase activity, or dNSF1, could serve to disassemble SNARE complexes, but our observation of severe physiological phenotypes suggests that $dNSF2^{E/Q}$ disrupts synaptic function independent of the SNARE complex cycle.

Interestingly, temperature-dependent inhibition of dNSF1 ATPase activity in *comatose* mutants has not been shown. In a recent article, the *comatose* mutations were engineered into mammalian NSF and then tested in a Golgi-reassembly membrane fusion assay (Muller et al., 1999). As predicted, the mutation blocked reassembly in a temperature-dependent manner. However, analysis of the ATPase activity of the mutant protein unexpectedly revealed that the ATPase activity was abolished at the permissive temperature, as well as at the restrictive temperature. Thus, Golgi reassembly, and by extension neural activity in *comatose* flies, is supported by the mutant protein in the absence of ATPase activity at permissive temperatures. In subcellular fractionation studies we have recently shown that while dNSF1 is distributed among all fractions, it is enriched in a synaptic vesicle fraction (Mohtashami et al., 2001). Maintaining *comatose* flies at restrictive temperatures led to an irreversible translocation of dNSF1 from the cytosol and membrane fractions to a Triton X-100 insoluble fraction, whereas wild-type dNSF1 does not translocate at restrictive temperatures. Together, these two observations suggest that although the *comatose* mutations affect dNSF1 ATPase activity, the major effect of temperature, and thus on SNARE complex accumulation, is on subcellular localization.

In the present study we expressed a mutant NSF molecule in which the ATPase activity is significantly reduced and presumably inactivates a majority of the NSF oligomers. Because the physiological phenotypes we reported are consistent with the temperature-dependent effects previously reported for *comatose* mutants, and because Muller et al. (1999) showed that

comatose NSF proteins lack ATPase activity, even at permissive temperature, the simplest interpretation of both the *comatose* data and the present results is that the phenotypes result from a reduction in NSF availability.

Although SNARE complex dissolution is the most widely studied property of NSF, it could potentially carry out other tasks. For example, it has been recently demonstrated that NSF has an important functional interaction with glutamate receptors (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Another potential role could be a direct interaction with monomeric SNAREs (Hanson et al., 1995). Our electron microscopy data indicates that there is not a major decrease in the number of synaptic vesicles, while our physiological estimates show a dramatic reduction in the number of the releasable vesicles in larvae expressing *dNSF2^{E/Q}*. Together, these data suggest that there are fewer functional vesicles and point towards a potential defect in vesicle activation.

The physiological phenotypes we observed are identical to those we previously reported for *syntaxin* mutants (Stewart et al., 2000). When Syntaxin levels are reduced to 20% of normal, by combining hypomorphic and null alleles, transmitter release is severely impaired, Ca^{2+} cooperativity is reduced, and, importantly, the frequency of spontaneous release reduced. The latter observation distinguishes *syntaxin* mutants from *neural-synaptobrevin (n-syb)* mutants because hypomorphic alleles of *n-syb* do not show a reduction in spontaneous release, even when evoked release is severely impaired (Stewart et al. 2000). Therefore, the present results on *dNSF2^{E/Q}* phenotype the physiological results obtained from *syntaxin* mutants, and suggest that NSF may have a role promoting Syntaxin function. If this activity were disrupted in the present study, we would not expect to observe an increase in SNARE complex abundance, but would expect the physiological phenotypes, including a reduction in the number of functional vesicles, which we report.

Contrasting the similar physiological results obtained between *dNSF2^{E/Q}* and *syntaxin* mutants is the major change in synaptic morphology that occurs in the *dNSF2^{E/Q}* expressing larvae. There are no morphological changes in the *syntaxin* mutants (Stewart et al., 2000). This suggests that NSF has a role in synaptogenesis and raises the question: to what extent does the morphological phenotype contribute to the physiological phenotype?

There are many instances where the morphology of the *Drosophila* larval synapse is altered, and the effect on synaptic physiology has been characterized. Fasciclin II has been implicated as the major regulatory

molecule controlling synaptic morphology (Schuster et al., 1996a,b). Manipulation of Fasciclin II levels can cause either reduction or expansion of the NMJ with no change in synaptic strength (Stewart et al., 1996; Davis and Goodman, 1998). If the reverse approach is taken and synaptic transmission is impaired directly, for example with mutants of Synaptotagmin (DiAntonio and Schwarz, 1994) or the SNARE proteins (Stewart et al., 2000), there is no corresponding change in NMJ morphology. In mutants of the *dunce* gene there is an expansion of the NMJ with a corresponding increase in synaptic strength (Zhong and Wu, 1991). Mutants of the *highwire* gene cause synaptic overgrowth with a corresponding reduction in synaptic strength (Wan et al., 2000), similar to the result reported here. Thus, there is no strict correlation between NMJ morphology and synaptic transmission. Although we observe opposing effects on NMJ morphology and synaptic transmission in the *dNSF2^{E/Q}* larvae, it remains possible, though we think unlikely, that dilution of a rate-limiting synaptic component resulting from synaptic overgrowth contributes to the physiological phenotype we observed.

The recent discovery that NSF is a component of the presynaptic particle (Phillips et al., 2001) may provide a framework in which to reconcile the observations reported here. Cytomatrix proteins, such as Piccolo and Basoon, likely interact with proteins of the presynaptic particle, including NSF. Because the presynaptic particle is linked to the postsynaptic membrane by adhesive molecules, disruption of members of the presynaptic particle may reduce adhesion and thus be permissive to the overgrowth of the NMJ that we observe here. Further studies aimed at understanding the multiple functions of NSF within the presynaptic nerve terminal will yield valuable data on the role of NSF in synaptic transmission and development.

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