Interaction of Cytoskeleton Genes With NSF2-Induced Neuromuscular Junction Overgrowth

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Summary: N-Ethylmaleimide sensitive factor (NSF) is an ATPase whose activity is important for intracellular trafficking. Previous genetic analysis of Drosophila NSF2 revealed a potential link between NSF and the actin cytoskeleton. The present study was therefore undertaken to specifically examine genetic interactions between the cytoskeleton and NSF. First, we tested for loss-of-function interaction and, indeed, we found that the combination of flies heterozygous for Act5C and NSF2 alleles led to reduced viability. Second, we expanded our gain-of-function approach to include cytoskeletal genes that were not included in our previous screen. Thirteen of 30 genes tested were found to suppress neuromuscular junction (NMJ) overgrowth. Altogether, these data support the idea that diverse NSF2 developmental and physiological phenotypes are related to disruption of the cytoskeleton and the large number of genes which can partially restore NMJ overgrowth and suggests that NSF may function near the top of the actin regulatory pathway.

Key words: Drosophila; synapse; neuromuscular junction; actin; microtubules

To understand the developmental and physiological role of N-ethylmaleimide sensitive factor (NSF) at neural synapses, we have taken a genetic approach using Drosophila melanogaster as a model system. NSF is a member of the AAA ATPase family of proteins, and previous work has shown that ATP hydrolysis allows NSF to disassemble the SNARE complex of proteins. The data supporting this canonical role for NSF are extensively reviewed elsewhere (May et al., 2001; Whiteheart et al., 2001). It is also likely that NSF serves other roles in the cell (Whiteheart and Matveeva, 2004).

The Drosophila genome has two NSF encoding genes, comatose (which encodes NSF1) and NSF2 (Boulianne and Trimble, 1995; Ordway et al., 1994). Experiments by Golby et al. (2001) show that the two proteins are functionally interchangeable, but that normal biological constraints make NSF1 the predominant isoform in the adult fly central nervous system, whereas NSF2 is found to be active at earlier developmental stages and in a broader range of tissues. To take advantage of the accessible and well-characterized third larval instar neuromuscular junction (NMJ), we have used an engineered allele of NSF2 in which glutamate 326, within the D1 ATPase domain, was exchanged for glutamine (Stewart et al., 2001). This mutation was based upon similar studies of mammalian NSF; it reduces the hydrolytic activity of the molecule and acts as a dominant negative, since one mutated subunit is sufficient to reduce the ATPase activity of the NSF hexamer (Whiteheart et al., 1994). The developmental and physiological consequences of expressing this form of NSF2—called NSF2E/Q—in neurons have been previously reported (Stewart et al., 2002, 2005), and they include suppression of synaptic transmission, increased synaptic fatigue, a reduced number of Tbar active zones at synapses and, surprisingly, extensive overgrowth of the NMJ.

To understand the nature of the NMJ overgrowth phenotype, we carried out an unbiased gain-of-function suppressor screen to identify genes that could reduce the overgrowth (Laviolette et al., 2005). Among others, we identified nine genes that are components of, or have the potential to regulate, the actin cytoskeleton. This result is interesting because many of the previously reported phenotypes attributed to NSF2E/Q may be explained if we assumed that disruption of the cytoskeleton was a primary cause of the phenotypes. The disruption of actin was confirmed by the observation that there is less filamentous actin in the NSF2E/Q nerve terminal (Nunes et al., 2006). In turn, the result is novel since no previous report has linked NSF activity to the cytoskeleton.

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If the actin cytoskeleton is perturbed in NSF2E/Q flies, we reasoned that actin loss of function alleles should also genetically interact with the NSF2E/Q allele. Two genes encode cytoplasmic actin in Drosophila, Actin5C, and Actin42A. We previously recovered Actin5C as a gain of function suppressor, whereas Actin42A is not (see later). Further, Actin5C has been previously shown to be expressed in the nervous system (Tobin et al., 1990). We therefore crossed (Fig. 1a) Act5C^{PG28}/FM7, a lethal loss of function allele, to elav^{34}-Gal4::UAS-NSF2E/Q/TM3,Sb and recorded the number of viable Act5C^{PG28}/+; elav^{34}-Gal4::UAS-NSF2E/Q/+ and Act5C^{PG28}/+; TM3,Sb/+ flies. If there is no genetic interaction, the number of flies of these two genotypes should be equal. On the contrary, when we scored 208 female flies, we observed a 1:2 ratio of those carrying elav^{34}-Gal4::UAS-NSF2E/Q versus those carrying TM3,Sb (Fig. 1b). Thirty three of 218 females carried Act5C^{PG28} in combination UAS-NSF2E/Q, whereas 68/208 had Act5C^{PG28} in combination with TM3,Sb, and 50/208 and 57/208 flies had FM7 in combination with UAS-NSF2E/Q and TM3,Sb respectively (Fisher’s exact test, P = 0.002). These data suggest that the combination of Act5C and NSF2 alleles leads to a semilethal interaction.

To begin a targeted gain of function screen, we cross-referenced the Gene Ontology classification terms "structural component of the cytoskeleton" and "cytoskeleton organization," to the Gene Search (GS) database (http://gsdb.biol.metro-u.ac.jp/~dclust/). This identified 46 GS lines with transposons with potential to upregulate expression of genes associated with the cytoskeleton, which we had not tested in our previous screen. From this list, we selected 30 lines for further study. These lines and their ability to suppress NSF2E/Q-induced neuromuscular overgrowth are shown in Table 1. We did not test the remaining 16 lines because of potential confounding results arising from the insertion site of the GS transposon.

Selecting cytoskeleton-related genes was an effective strategy for identifying suppressors of NSF2E/Q-induced overgrowth, since 13 of the 30 lines yielded some suppression of this phenotype. Eighteen lines showed either weak or no suppression, and we did not characterize these lines further.

We next carried out a secondary screen of dissected third instar larval NMJs by immunohistochemistry (Fig. 2). The number of rescued NMJs was recorded for each genotype from an examination of the muscle 6/7 and muscle 12/13 synapses of abdominal segments 2, 3, and 4 from a minimum of 4 larvae. In total, we examined 884 neuromuscular synapses from 74 dissected larvae. We defined a rescued NMJ as one in which the nerve terminal was obviously shorter and the bouton size larger than in the NSF2E/Q NMJs (Fig. 2a). The percentage of rescued NMJs is shown in Figure 2b. It should be noted that all of these GS lines passed our initial screen and that this more rigorous classification further delineates the most effective suppressors.

From Figure 2 it is clear that four lines provided the most consistent rescue among the 12 tested. They are Gs7380 (moestin), Gs13418 (quail), Gs51423 (jaguar), and Gs51783 (β-tubulin56D). One other line, Gs16851 (still life), rescued an intermediate number of junctions, while the remainder of GS lines rescued a more limited number of nerve terminals.

We also tested each of these GS lines in combination with elav^{34}-Gal4 to determine whether gene expression controlled by these transposons effects NMJ morphology in the wild-type background. We did not find any obvious change in NMJ shape or size when these GS lines were expressed with elav-Gal4 alone (Fig. 3). In particular, we looked for undergrowth of the NMJs to address the possibility that suppression of the NSF2E/Q phenotype is an additive effect; however, we observed no such change in NMJ morphology. This suggests that the observed rescue is relevant to the NSF2E/Q-induced overgrowth rather than being a nonspecific additive effect on NMJ shape.

Three of the GS lines that most reliably rescue the overgrowth phenotype are consistent with our previous findings that perturbation of the actin cytoskeleton is a
GS lines tested which show weak or no suppression of NSF2<sup>E/Q</sup>-induced NMJ overgrowth.

**Table 1**

<table>
<thead>
<tr>
<th>GS line</th>
<th>Drosophila gene name</th>
<th>Protein family or function</th>
</tr>
</thead>
<tbody>
<tr>
<td>51783</td>
<td>β-tubulin56D</td>
<td>Component of microtubules</td>
</tr>
<tr>
<td>51423</td>
<td>jaguar</td>
<td>Myosin VI homolog</td>
</tr>
<tr>
<td>50262</td>
<td>α-spectrin</td>
<td>Actin crosslinking protein</td>
</tr>
<tr>
<td>50077</td>
<td>zipper</td>
<td>Nonmuscle myosin II</td>
</tr>
<tr>
<td>21904</td>
<td>β-tubulin56D</td>
<td>Component of microtubules</td>
</tr>
<tr>
<td>21857</td>
<td>didum</td>
<td>Dilute class unconventional myosin (myosin V)</td>
</tr>
<tr>
<td>17924</td>
<td>Myosin heavy chain-like&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Myosin XVIII</td>
</tr>
<tr>
<td>16581</td>
<td>still life</td>
<td>Rho guanyl-nucleotide exchange factor activity</td>
</tr>
<tr>
<td>14898</td>
<td>microtubule-associated protein 205</td>
<td>Microtubule binding protein</td>
</tr>
<tr>
<td>14528</td>
<td>synapsin</td>
<td>Linker between vesicles and actin</td>
</tr>
<tr>
<td>13418</td>
<td>quail</td>
<td>Villin-like protein</td>
</tr>
<tr>
<td>9302</td>
<td>genghis khan</td>
<td>Putative Cdc42 regulator</td>
</tr>
<tr>
<td>7380</td>
<td>moesin</td>
<td>ERM family member linking actin to membranes</td>
</tr>
</tbody>
</table>

**GS lines tested which show weak or no suppression**

<table>
<thead>
<tr>
<th>GS line</th>
<th>Drosophila gene name</th>
<th>Protein family or function</th>
</tr>
</thead>
<tbody>
<tr>
<td>50849</td>
<td>chickadee</td>
<td>Profilin homolog</td>
</tr>
<tr>
<td>50418</td>
<td>Arc-p34</td>
<td>Component of Arp2/3 protein complex</td>
</tr>
<tr>
<td>22446</td>
<td>twinfilin</td>
<td>Actin monomer-binding protein</td>
</tr>
<tr>
<td>21982</td>
<td>canoe</td>
<td>Potential actin ras binding protein</td>
</tr>
<tr>
<td>21746</td>
<td>α-tubulin 84D</td>
<td>Component of microtubules</td>
</tr>
<tr>
<td>20877</td>
<td>Netrin A</td>
<td>Signaling molecule</td>
</tr>
<tr>
<td>20622</td>
<td>twinstar</td>
<td>ADF/cofilin homolog</td>
</tr>
<tr>
<td>17911</td>
<td>Actin42A</td>
<td>Cytoplasmic actin</td>
</tr>
<tr>
<td>17416</td>
<td>dynemin light chain 90F</td>
<td>Component of dynemin microtubule associated complex</td>
</tr>
<tr>
<td>17247</td>
<td>twinstar</td>
<td>ADF/cofilin homolog</td>
</tr>
<tr>
<td>16080</td>
<td>twinstar</td>
<td>ADF/cofilin homolog</td>
</tr>
<tr>
<td>15493</td>
<td>CGT0540</td>
<td>Potential actin barbed-end capping protein</td>
</tr>
<tr>
<td>15487</td>
<td>fat facets&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Deubiquitinating protease</td>
</tr>
<tr>
<td>14652</td>
<td>sallimus</td>
<td>Cytoskeletal component of muscle</td>
</tr>
<tr>
<td>14012</td>
<td>thread</td>
<td>Ubiquitin-protein ligase activity</td>
</tr>
<tr>
<td>13234</td>
<td>thread</td>
<td>Ubiquitin-protein ligase activity</td>
</tr>
<tr>
<td>12660</td>
<td>microtubule-associated protein 60</td>
<td>Microtubule binding protein</td>
</tr>
</tbody>
</table>

<sup>a</sup>Found to suppress in the primary screen but not analyzed in the secondary screen.

<sup>b</sup>Few larvae were recovered that coexpress NSF2<sup>E/Q</sup> and GS15487 indicating a potential lethal interaction.

**Table 1** GS Lines and Associated Genes Tested for Suppression of NSF2<sup>E/Q</sup>-Induced NMJ Overgrowth

Feature underlying the NSF2<sup>E/Q</sup> phenotype. Moesins are members of the ERM family, which are actin-binding proteins that link the cytoskeleton to the plasma membrane. We previously identified moesin in our first, unbiased, screen of the GS collection, albeit with a different GS insertion. This result confirms the previous observation.

Jaguar encodes the Drosophila homolog of Myosin VI. The MyoVI group are unconventional cytoplasmic myosins whose precise role in cell biology is not known. A recent model for the interaction of MyoVI with actin suggests that this molecule has a role in stabilizing actin filaments (Frank et al., 2004; Rogat and Miller, 2002). This latter function fits with our previous findings that expression of proteins with the capability to stabilize actin rescues the NSF2<sup>E/Q</sup> phenotype.

Quail is a gene which encodes a villin-like protein that has actin bundling activity (Mahajan-Miklos and Cooley, 1994). No report has previously indicated a role for quail in the Drosophila nervous system, although similar proteins in other species have been shown to be important in neural development, through interactions with actin (Lundquist et al., 1998; Ravenall et al., 2002).

Still life (sif) encodes a Rac guanine-nucleotide exchange factor (Sone et al., 1997, 2000) that potentially activates Rho by stimulating the exchange of GDP for GTP. Members of this small GTPase family are well known to play a role in actin filament biochemistry (Hall, 1998; Luo, 2000; Nikolic, 2002). Furthermore, genetic analysis of sif shows that a loss of function mutant has a reduced number of nerve terminal boutons, whereas overexpression of sif had no effect on nerve terminal morphology (Sone et al., 2000).

Our finding here that coexpression of β-tubulin suppresses the NSF2<sup>E/Q</sup>-induced overgrowth is the only indication so far that this component of the cytoskeleton may be involved in the NSF2<sup>E/Q</sup> phenotype. This positive result is contrasted by the lack of effect of a GS insertion positioned at the 5' end of the α-tubulin gene. Owing to the complex nature of the interaction between microtubules and F-actin (Rodriguez et al., 2003), interpretation of this result is difficult. It is interesting to note, however, that in some cases microtubules have been shown to be important for F-actin polymerization (Grabham et al., 2003; Rochlin et al., 1999) in neural growth cones.

We therefore examined the nerve terminals of control and NSF2<sup>E/Q</sup> larvae for microtubules (Fig. 4). From these experiments, we could clearly identify microtubules in the axons extending into the nerve terminal boutons of...
both genotypes. Furthermore, a feature of some boutons is the appearance of microtubule loops (Roos et al., 2000), and we could readily identify these in both genotypes as well. This observation suggests, to a first approximation, that the microtubule cytoskeleton is not severely damaged in the NSF2e/Q nerve terminals in contrast to the obvious disruption of the actin cytoskeleton we previously observed (Nunes et al., 2006). When we counted the number of boutons that contained a microtubule loop, we found that yw controls had 20.3 ± 1.8 boutons per NMJ with a loop (n = 16 NMJs from six larvae) and that NSF2e/Q samples showed 23.5 ± 2.9 boutons per NMJ with a loop (n = 16 NMJs from six larvae). There is no statistical difference between the genotypes (P > 0.05, t-test). Altogether, given the normal appearance of microtubules and the apparent normal delivery of microtubule-dependent synaptic vesicles to the nerve terminal (Stewart et al., 2005), we presently favor the idea that overexpression of β-tubulin likely rescues the NSF2e/Q phenotype because of an interaction with the actin cytoskeleton.

In summary, we have carried out a loss-of-function test and gain-of-function screen to further investigate the previous finding that perturbation of the cytoskeleton is

FIG. 2. (a) GS expression of cytoskeletal genes reveals suppression of elav-Gal4:UAS-NSF2e/Q-induced NMJ overgrowth. (a1) Normal NMJ morphology from the yw control genotype. (a2) NMJ overgrowth typically found in elav-Gal4:UAS-NSF2e/Q larvae. (a3–a6) show examples of rescued NMJ morphology by GS lines. (a3) shows rescue by GS13428 (quail); (a4) shows rescue by GS16851 (sif); (a5) shows rescue by GS51423 (jag); (a6) shows rescue by GS51783 (β-tubulin56D). Arrowheads point to the muscle 6/7 NMJ. (b) Quantification of the number of NMJs rescued by GS lines. Muscle 6/7 and muscle 12/13 NMJs were scored for rescue as described in the text and are presented as a percentage of the total number of NMJs examined for each GS line.

FIG. 3. Neural expression of the GS lines alone does not change NMJ morphology. The yw control strain is shown for comparison (same as Fig. 2a); in each subsequent image, the complete genotype is elav-Gal4 × the GS line indicated on the panel. In all cases the NMJ morphology appears identical to the control strain.
a major factor contributing to NSF2E/Q-induced NMJ overgrowth. Both of these tests support the previous result. The growing number of cytoskeletal interacting proteins that restore the NSF2E/Q-induced NMJ overgrowth likely indicates that the lesion which leads to the phenotype is high up in the actin regulatory pathway. Further delineation of this phenotype will require complementary loss-of-function genetic analysis, as well as biochemical tests for novel NSF binding partners. A recent paper (Martin et al., 2006) reports that betaPix, a guanine exchange factor that activates p21-activated kinase (PAK), physically interacts with NSF. Because PAKs have the well-known ability to influence actin biochemistry (Eby et al., 1998), this finding suggests a novel route by which NSF may regulate the cytoskeleton.

MATERIALS AND METHODS

Drosophila Stocks and Genetics
All crosses were carried out at 25°C, and stocks were maintained on Bloomington standard medium (http://flystocks.bio.indiana.edu/bloom-food.htm).

Immunocytochemistry
In order to confirm the observations made in the intact larvae, we performed secondary screening by staining the NMJ. Third instar larvae were dissected in HL3 saline (Stewart et al., 1994), then fixed in 4% formaldehyde for 10 min, washed in phosphate buffered saline plus 0.1% methanol.

FIG. 4. Examination of microtubules in the elav-Gal4:UAS-NSF2E/Q nerve terminal. NMJs were labeled with anti-HRP, a general neural membrane marker (green) and anti-acetylated tubulin (red). (a) In control yw samples, microtubules can be easily detected in the nerve terminal as long thin process that propagate through the nerve terminal branches, and occasionally "looped" structures are observed within boutons. (b) In elav-Gal4:UAS-NSF2E/Q samples, microtubules can similarly be seen in the nerve terminal as long thin process and "loops" are also readily identified.
Triton X-100 for 30 min, followed by a 1–2 h incubation at room temperature, or overnight at 4°C, in 1:1,000 dilution of FITC-conjugated anti-HRP antibody (ICN Biochemicals). The preparations were washed for a further 30 min and then mounted in Vectashield (Vector Labs) for microscopic analysis. Images were acquired on a Zeiss LSM 510 confocal by collecting z-sections at 1-μm intervals and projecting the images onto a single plane.

Microtubules were examined at the NMJ using mouse anti-acetylated α-tubulin (Sigma), at a dilution of 1:1,000, with the above protocol, with the exception that the dissected preparations were fixed for 1 h. The secondary antibody, goat anti-mouse Alexa594, was used at 1:500 (Molecular Probes).

Statistical tests were done using Prism4.0 (Graphpad) with P < 0.05 as the level of significance.

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LITERATURE CITED


Martin HG, Henley JM, Meyer G. 2006. Novel putative targets of N-ethylmaleimide sensitive fusion protein (NSF) and α/β soluble NSF attachment proteins (SNAPs) include the Pak-binding nucleotide exchange factor βPIX. J Cell Biochem 99:1203–1215.


Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. 1994. Improved sta-


