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LETTER

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. genesis 44:595–600, 2006. © 2006 Wiley-Liss, Inc.

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 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 $NSF2^{E/Q}$—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 T-bar 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 $NSF2^{E/Q}$ 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 $NSF2^{E/Q}$ 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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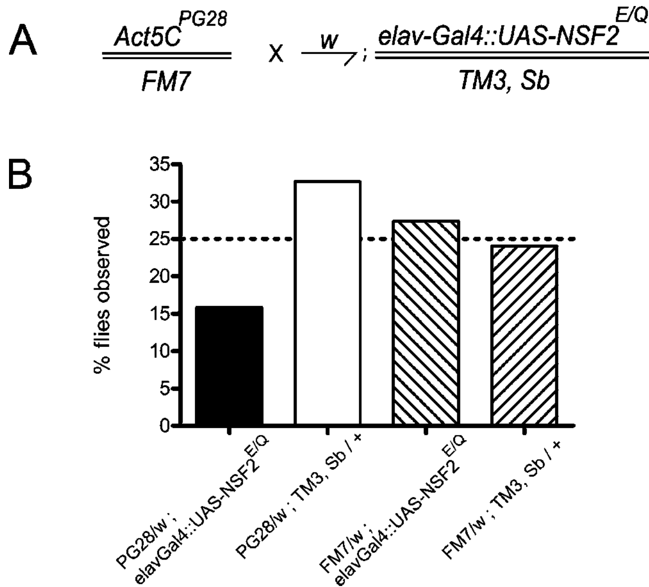

FIG. 1. Interaction between *Actin5C* loss of function and *elav-Gal4:UAS-NSF2*$^{E/Q}$. (**a**) Genetic scheme to test for genetic interaction between heterozygous *Actin5C*^{PG28} females and neurally expressed *UAS-NSF2*$^{E/Q}$. (**b**) Percentage of flies recovered. The total number of females counted was 208. The dashed line indicates the expected value of 25%.

We therefore undertook the present targeted experiments to determine whether we could identify other important cytoskeletal elements that may be contributing to the *NSF2*$^{E/Q}$ overgrowth phenotype.

RESULTS AND DISCUSSION

If the actin cytoskeleton is perturbed in *NSF2*$^{E/Q}$ flies, we reasoned that actin loss of function alleles should also genetically interact with the *NSF2*$^{E/Q}$ 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*^{3A}*-Gal4:UAS-NSF2*$^{E/Q}$*/TM3,Sb* and recorded the number of viable *Act5C*^{PG28}*/+; elav*^{3A}*-Gal4:UAS-NSF2*$^{E/Q}$*/+* 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*^{3A}*-Gal4:UAS-NSF2*$^{E/Q}$ versus those carrying *TM3,Sb* (Fig. 1b). Thirty three of 218 females carried *Act5C*^{PG28} in combination *UAS-NSF2*$^{E/Q}$, whereas 68/208 had *Act5C*^{PG28} in combination with *TM3,Sb*, and 50/208 and 57/208 flies had *FM7* in combination with *UAS-NSF2*$^{E/Q}$ 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 *NSF2*$^{E/Q}$ 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 *NSF2*$^{E/Q}$-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 NSF2$^{E/Q}$ 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 (*moesin*), 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*^{3A}*-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 *NSF2*$^{E/Q}$ phenotype is an additive affect; however, we observed no such change in NMJ morphology. This suggests that the observed rescue is relevant to the *NSF2*$^{E/Q}$-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

Table 1
GS Lines and Associated Genes Tested for Suppression of NSF2$^{E/Q}$*-Induced NMJ Overgrowth*

GS line	*Drosophila* gene name	Protein family or function
Potential suppressors		
51783	*β-tubulin56D*	Component of microtubules
51423	*jaguar*	Myosin VI homolog
50262	*α-spectrin*	Actin crosslinking protein
50077	*zipper*	Nonmuscle myosin II
21904	*β-tubulin56D*	Component of microtubules
21857	*didum*	Dilute class unconventional myosin (myosin V)
17924	*Myosin heavy chain-like*[a]	Myosin XVIII
16581	*still life*	Rho guanyl-nucleotide exchange factor activity
14898	*microtubule-associated protein 205*	Microtubule binding protein
14528	*synapsin*	Linker between vesicles and actin
13418	*quail*	Villin-like protein
9302	*genghis khan*	Putative Cdc42 regulator
7380	*moesin*	ERM family member linking actin to membranes
GS lines tested which show weak or no suppression		
50849	*chickadee*	Profilin homolog
50418	*Arc-p34*	Component of Arp2/3 protein complex
22446	*twinfilin*	Actin monomer-binding protein
21982	*canoe*	Potential actin ras binding protein
21746	*α-tubulin 84D*	Component of microtubules
20877	*Netrin A*	Signaling molecule
20622	*twinstar*	ADF/cofilin homolog
17911	*Actin42A*	Cytoplasmic actin
17416	*dynein light chain 90F*	Component of dynein microtubule associated complex
17247	*twinstar*	ADF/cofilin homolog
16080	*twinstar*	ADF/cofilin homolog
15493	*CG10540*	Potential actin barbed-end capping protein
15487	*fat facets*[b]	Deubiqunniating protease
14652	*sallimus*	Cytoskeletal component of muscle
14012	*thread*	Ubiquitin-protein ligase activity
13234	*thread*	Ubiquitin-protein ligase activity
12660	*microtubule-associated protein 60*	Microtubule binding protein

[a]Found to suppress in the primary screen but not analyzed in the secondary screen.
[b]Few larvae were recovered that coexpress NSF2$^{E/Q}$ and GS15487 indicating a potential lethal interaction.

feature underlying the *NSF2$^{E/Q}$* 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$^{E/Q}$* 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$^{E/Q}$*-induced overgrowth is the only indication so far that this component of the cytoskeleton may be involved in the *NSF2$^{E/Q}$* 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$^{E/Q}$* larvae for microtubules (Fig. 4). From these experiments, we could clearly identify microtubules in the axons extending into the nerve terminal boutons of

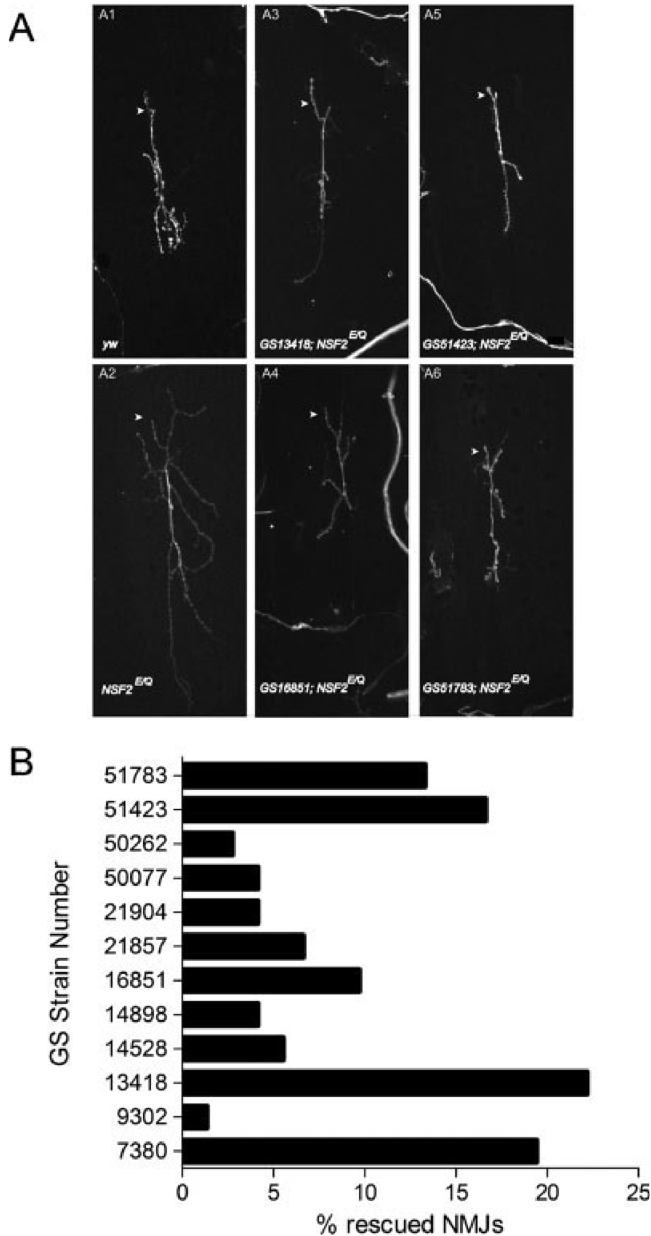

FIG. 2. (**a**) GS expression of cytoskeletal genes reveals suppression of *elav-Gal4:UAS-NSF2$^{E/Q}$*-induced NMJ overgrowth. (**a1**) Normal NMJ morphology from the *yw* control genotype. (**a2**) NMJ overgrowth typically found in *elav-Gal4:UAS-NSF2$^{E/Q}$* larvae. (**a3–a6**) show examples of rescued 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.

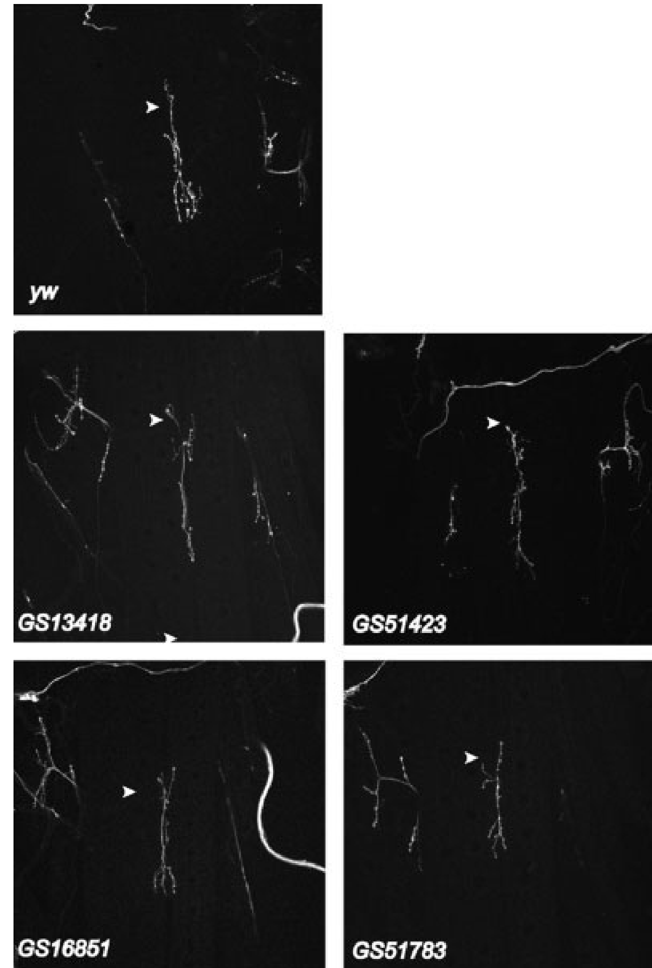

FIG. 3. Neural expression of the GS lines alone does not change NMJ morphology. The *yw* control strain is shown for comparison (same as Fig. 2a); in each subsequent image, the complete genotype is *elav-Gal4* × the GS line indicated on the panel. In all cases the MNJ morphology appears identical to the control strain.

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 *NSF2$^{E/Q}$* 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 *NSF2$^{E/Q}$* 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 *NSF2$^{E/Q}$* 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

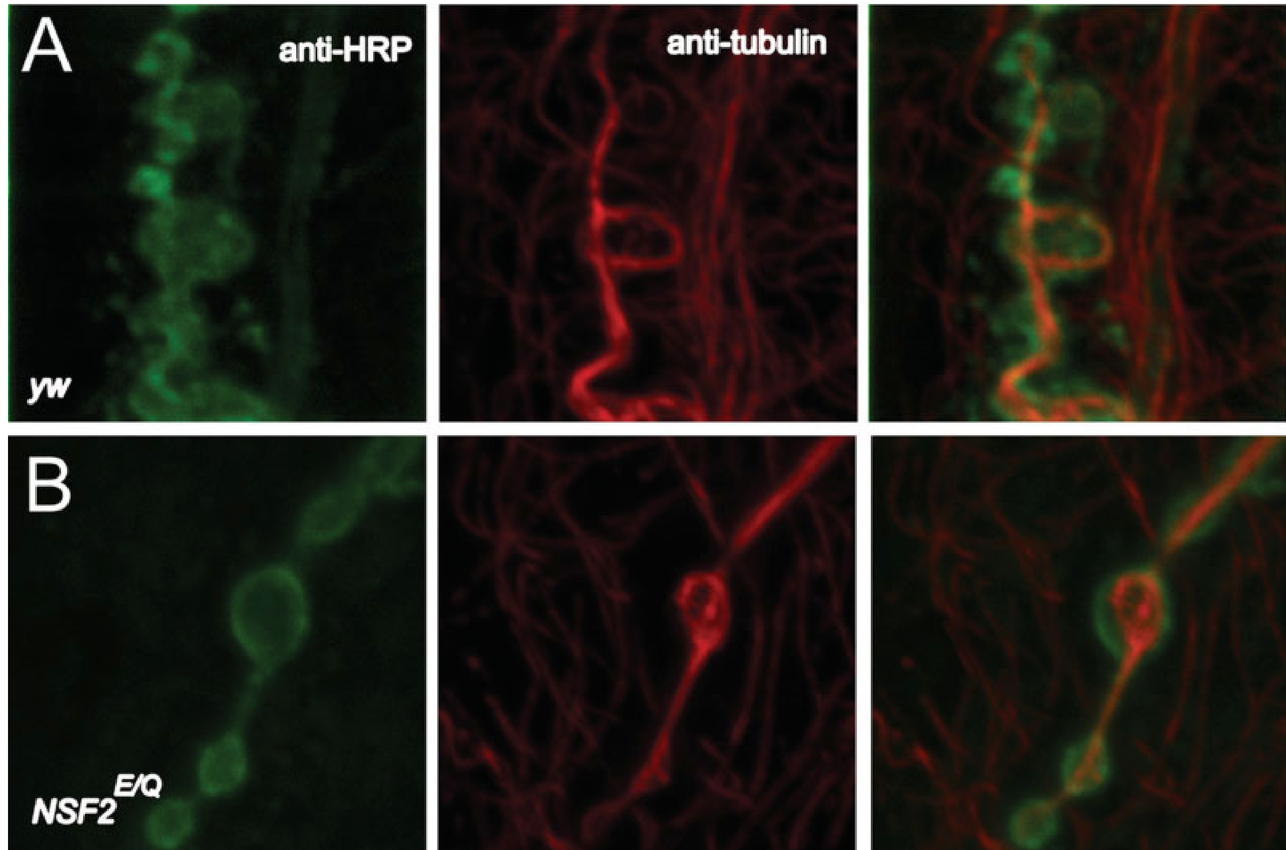

FIG. 4. Examination of microtubules in the *elav-Gal4:UAS-NSF2*$^{E/Q}$ nerve terminal. NMJs were labeled with anti-HRP, a general neural membrane marker (green) and anti-acetylated tubulin (red). (**a**) In control *yw* samples, microtubules can be easily detected in the nerve terminal as long thin process that propagate through the nerve terminal branches, and occasionally "looped" structures are observed within boutons. (**b**) In *elav-Gal4:UAS-NSF2*$^{E/Q}$ samples, microtubules can similarly be seen in the nerve terminal as long thin process and "loops" are also readily identified.

a major factor contributing to *NSF2*$^{E/Q}$-induced NMJ overgrowth. Both of these tests support the previous result. The growing number of cytoskeletal interacting proteins that restore the *NSF2*$^{E/Q}$-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 complimentary 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).

The present screen was carried out as described in Laviolette *et al.* (2005). In brief, we crossed five to 10 virgin females of the tester stock *w Mhc.CD8-GFP-Sh; elav*^{3A}*-Gal4.UAS-NSF2*$^{E/Q}$*/TM6B,Tb* to 3–5 males from GS lines. Non-*Tb* larval offspring were selected for analysis. To visualize the NMJ in the intact larvae, we immobilized larvae by placing them into a 0.2-mL tube with 100 μL of glycerol, which was heated to 60°C for 10 s in a thermocycler. GFP signals were visualized through the larval cuticle at the NMJ with a 40× air lens on a fluorescence compound microscopy. For the initial screen, we scored NMJs of muscles 12 and 13, because they are the easiest muscles to visualize through the intact cuticle.

Act5C^{PG28} is a lethal P-element insertion into the *Actin5C* gene described in Bourbon *et al.* (2002).

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%

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 goat 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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