Cellular/Molecular

Drosophila Amphiphysin Functions during Synaptic Fasciclin II Membrane Cycling

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Recent studies have revealed that endocytosis and exocytosis of postsynaptic receptors play a major role in the regulation of synaptic function, particularly during long-term potentiation and long-term depression. Interestingly, many of the proteins implicated in exocytosis and endocytosis of synaptic vesicles are also involved in postsynaptic protein cycling. In vertebrates, Amphiphysin is postulated to function during endocytosis in nerve terminals; however, several recent reports using a *Drosophila amphiphysin* (*damph*) null mutant have failed to substantiate such a role at fly synapses. In addition, Damph is surprisingly enriched at the postsynapse. Here we used the glutamatergic larval neuromuscular junction to study the synaptic role of Damph. By selectively labeling internal and external pools of the cell adhesion molecule Fasciclin II (FasII), and by using a novel *in vivo* surface FasII immunocapture protocol, we show that the level of external FasII is decreased in *damph* mutants although the total level of FasII remains constant. *In vivo* FasII internalization assays indicate that the reincorporation of FasII molecules into the cell surface is severely inhibited in *damph* mutants. Moreover, we show that blocking soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) function in postsynaptic muscle cells interferes with FasII exocytosis. These experiments suggest that in *Drosophila,* Damph functions during SNARE-dependent postsynaptic FasII membrane cycling. This study challenges the notion that synaptic Amphiphysin is involved exclusively in endocytosis and suggests a novel role for this protein in postsynaptic exocytosis.

Key words: **Amphiphysin; Fasciclin II; exocytosis; postsynapse; SNARE; plasticity**

Introduction

Exocytosis at the synapse is generally viewed as a process for the release of neurotransmitter-containing vesicles from the presynaptic terminal (Slepnev and De Camilli, 2000). Recent studies, however, suggest that postsynaptic exocytotic mechanisms can regulate the availability of neurotransmitter receptors at the postsynaptic membrane. For example, at vertebrate central synapses, endocytosis of AMPA-type receptors underlies, at least in part, the decrease in synapse strength observed during long-term depression (LTD) (Ehlers, 2000; Carroll et al., 2001). Similarly, AMPA-type glutamate receptors can be integrated into the postsynaptic membrane by exocytosis in an activity-dependent manner, contributing to an activity-dependent increase in synapse strength (Passafaro et al., 2001; Sheng and Kim, 2002).

During the last several decades many of the players that mediate exocytosis and endocytosis of synaptic vesicles have been identified. In these processes, the *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and Munc-18 appear to be central for exocytosis (Rizo and Sudhof, 2002), and endocytosis of synaptic vesicles is accomplished primarily by a Clathrin-dependent mechanism (Slepnev and De Camilli, 2000). Amphiphysin I (Amph I) was recently added to the host of proteins already known to function during endocytosis (Slepnev and De Camilli, 2000). Additional evidence supports a role for Amph I in the fission of Clathrin-coated vesicles by targeting the GTPase Dynamin to the coat (Shupliakov et al., 1997; Slepnev and De Camilli, 2000; Zhang and Zelhof, 2002). *In vitro* studies with liposomes show that purified Amph I has the ability to evaginate liposomes forming narrow tubes, raising the interesting possibility that Amph cooperates in the generation of highly curved membranes during vesicle fission (Slepnev and De Camilli, 2000).

Amph I is enriched at presynaptic terminals consistent with its putative function; however, an alternative Amph isoform, Amph II, has a ubiquitous expression. Amph II is particularly enriched in skeletal muscles, where it is involved in the genesis of T-tubules (Lee et al., 2002).

In fruit flies, a single Amphiphysin gene (*damph*) is expressed both at the muscle T-tubule network and the postsynaptic membrane of the neuromuscular junction (NMJ) (Razzaq et al., 2001). Null mutations in *damph* have abnormal T-tubules; however, consistent with its absence from presynaptic terminals but contrary to the reports from vertebrates, endocytosis of synaptic vesicles is normal in these mutants (Leventis et al., 2001; Razzaq et al., 2001; Zelhof et al., 2001). To understand the role of postsynaptic Damph at *Drosophila* synapses, we examined *damph* mu-

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tants for the distribution of synaptic proteins that normally colocalize with Damph at larval NMJs. In particular, we centered on the analysis of the cell adhesion molecule Fasciclin II (FasII), which plays a crucial role in the maintenance and plasticity of these glutamatergic synapses (Schuster et al., 1996a,b; Zito et al., 1999).

In this study, we examined the subcellular localization of the intracellular and transmembrane FasII pools in wild-type and *damph* null mutants. We further used *in vivo* internalization assays and FasII immunocapture to determine the role of Damph in this process. Our experiments demonstrate that in *Drosophila,* Damph plays a prominent role in FasII membrane cycling at the postsynaptic membrane, likely through a SNARE-dependent mechanism. These findings establish a novel role for Damph at synapses, particularly in the integration of postsynaptic membrane proteins.

Materials and Methods

Fly stocks. We used the following strains, which were reared at 25°C: wild type (Canton-S), the null mutant *damph*^{26} [from C. Doe (Zelhof et al., 2001), University of Oregon]; a deficiency for the *damph region, df (2R)vg-C* (Bloomington Stock Center at Indiana University); Gal4-responsive UAS stocks: UAS-Amph, containing a full-length form of *Drosophila* Amphiphysin (from C. Doe, University of Oregon), UAS-TNT-G, containing a tetanus toxin light chain transgene (Sweeney et al., 1995), UAS-IMPTNT containing an inactive form of TNT (from C. O'Kane, University of Cambridge), UAS-d-NSF-2$^{E/Q}$ containing a dominant-negative form of *N*-ethylmaleimide-sensitive fusion protein (NSF) (Stewart et al., 2002) (from B. Stewart, University of Toronto); and a muscle-specific GAL4 line: BG487 (Budnik et al., 1996).

Immunocytochemistry and antibodies. The Fas(ex) anti-peptide polyclonal antibody was generated against amino acids 737–751 (GIDVIQVAERQVFSS) of transmembrane Fasciclin II and affinity purified by Pocono Rabbit Farm and Laboratories (Canadensis, PA). Bouton number in segment A3 was assessed by counting anti-HRP-immunoreactive varicosities on muscles 6 and 7. Muscle surface areas were not significantly different in all genotypes analyzed. For Fas(ex) immunocytochemistry, samples were fixed for 30 min in 4% paraformaldehyde fixer [0.1 M phosphate buffer (PB), pH 7.2, 4% paraformaldehyde], washed in PB, incubated with the Fas(ex) antibody (1:2000) for 2 hr, postfixed for 5 min in 4% paraformaldehyde, permeabilized with PBT (PB, 0.2% Triton X-100), and incubated overnight with anti-Fasciclin II monoclonal antibody (1:2) [FasIImAb;1D4 (Seeger et al., 1993); Developmental Studies Hybridoma Bank, University of Iowa]. No staining was observed with the FasIImAb when applied under nonpermeabilized conditions. The Damph antibody (Razzaq et al., 2000) (from C. O'Kane, University of Cambridge) was used at 1:200 in permeabilized tissue. Fluorescently conjugated mouse and rabbit secondary antibodies (Jackson Labs, Bar Harbor, ME) were used at 1:200. Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Comparisons between genotypes were performed in samples processed simultaneously and by using the same confocal acquisition parameters using a Zeiss laser scanning microscope confocal microscope. Fluorescence signal intensity quantifications were performed using Zeiss PASCAL (version 3.0) image analysis software (Oberkochen, Germany). Briefly, the immunolabeled region surrounding individual boutons was traced manually, and the mean relative intensity (on a scale of 0–256) of staining across the selected area was measured automatically by the software and logged into Excel (Microsoft, Redmond, WA). The number of boutons used for measurement is indicated in the respective figure legends.

Internalization studies and immunocapture. To stain the surface FasII pool, larvae were dissected in Stewart's saline (SS) (Stewart et al., 1994) containing (in mM): 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 115 sucrose, 5 Trehalose, 5 mM HEPES, pH 7.2, containing 0.1 mM Ca^{2+}, and incubated for 1 hr with anti-Fas(ex) (diluted at 1:500 in SS containing 2 mM Ca^{2+}). Then samples were washed for 1 hr in SS containing 2 mM Ca^{2+} and fixed for 10 min in 4% paraformaldehyde. After samples were washed with PB, they were incubated for 1 hr with Alexa 647-conjugated chicken anti-rabbit secondary antibody (1:200 dilution), washed, and postfixed with 4% paraformaldehyde fixer for 10 min. To stain the internalized pool of FasII, the same preparations were then permeabilized with PBT and incubated for 1 hr with FITC-conjugated donkey anti-rabbit secondary antibody (1:200) along with Texas Red-conjugated goat anti-HRP (1:100) to label the presynaptic membrane. To eliminate the possibility that the FITC-conjugated antibody might bind to unoccupied sites of surface (noninternalized) FasII–Fas(ex) antibody complexes, we incubated the samples with the FITC-conjugated secondary antibody after incubation with the Alexa 647-conjugated antibody and before permeabilization. No signal was obtained in the green channel, thus confirming that surface FasII is labeled exclusively by the Alexa 647-conjugated secondary antibody and internalized FasII is labeled by the FITC-conjugated secondary antibody. To determine the time required for FasII cycling at the surface, internalization assays were performed as above, but the Fas(ex) antibody incubation was performed in the cold (4°C) for 2 hr followed by shifting of the samples to room temperature (RT) for the required amounts of time (5, 10, 15, and 60 min). For phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma, St. Louis, MO) treatment, samples were incubated simultaneously with 0.05 U of the enzyme (Wright and Copenhaver, 2000) and Fas(ex) antibody at 1:500 in SS containing 2 mM Ca^{2+}. All incubations were performed at RT except when mentioned otherwise.

In vivo immunocapture of surface FasII was performed by individually incubating 20 third-instar body wall muscle preparations per genotype with anti-Fas(ex) antibody (1:500) diluted in SS after dissection in SS containing 0.1 mM Ca^{2+}. After washes with 0.1 M PB, body wall muscles were homogenized in a standard radioimmunoprecipitation buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), and the extract was incubated for 2 hr with Protein A-coated Sepharose beads (Sigma). Bound and unbound fractions were loaded separately onto an 8% SDS gel. Western blotting was performed using anti-FasII (1:1000) and anti-tubulin (1:10,000). Quantification of band intensities was performed by first scanning the radiographic film on a linear response scanner (UMAX-Powerlook III; UMAX, Dallas, TX). The intensity of the bands was measured by importing the scanned images into the PASCAL software and by performing a mean densitometric analysis.

Statistical analysis. SPSS software (version 11.5) (SPSS Inc., Chicago, IL) was used for statistical analysis. One-way ANOVA tests were performed to assess significance levels with a 95% interval of confidence. Numbers represent mean ± SEM.

Results

Levels of membrane-surface FasII are decreased in *damph* mutants

To study the function of Damph at *Drosophila* synapses, we used the third-instar larval NMJ, a powerful model system to study glutamatergic synapse development and function. At these synapses, Damph localizes primarily at the postsynaptic region in colocalization with a number of synaptic proteins, including FasII (Fig. 1*a–c*). Interestingly, it has been shown previously that changes in levels of FasII are correlated with the process of bouton budding, which is responsible for NMJ expansion during muscle growth (Zito et al., 1999). In severe hypomorphic *fasII* mutants, the synaptic bouton number is reduced considerably (Stewart et al., 1996), and we found a similar phenotype in *damph* null mutants [79.5 ± 6.9 boutons in wild-type ($n = 10$), 51.2 ± 6.4 in *fasII*^{e76} ($n = 10$), and 50.8 ± 7.9 in *damph* mutants ($n = 10$)]. Bouton numbers were further decreased to 30.1 ± 4.8 in *damph;fasII* double mutants ($n = 10$), but this reduction was close to that expected from an additive effect (expected number of boutons from an additive effect is 31.8), and therefore we could not conclude that *damph* and *fasII* interact genetically.

To determine whether the reduction in bouton number observed in *damph* mutants correlated with a corresponding reduc-

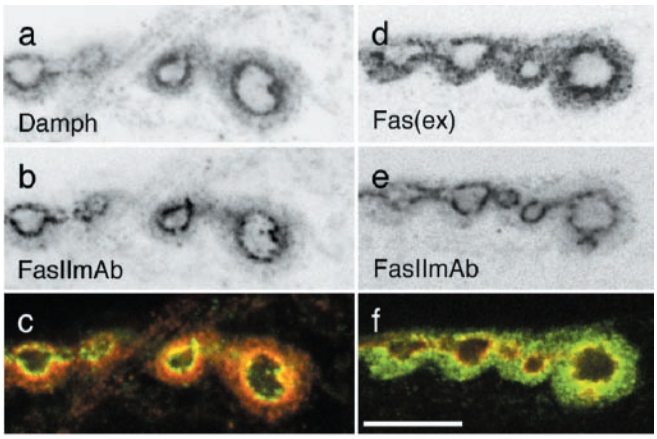

Figure 1. FasII and Damph colocalization and Fas II distribution at synaptic boutons. *a–c*, Single confocal slices of NMJs from wild-type third-instar larvae double labeled with anti-Damph (*a*, red) and anti-FasIImAb (*b*, green). In *c*, the above images have been merged. *d–f*, Single confocal slices of NMJs from wild-type third-instar larvae double labeled with an antibody against a FasII extracellular epitope [anti-Fas(ex)] (*d*, green) and a monoclonal antibody against the intracellular C-terminal tail of FasII (*e*, red). In *f* the above images have been merged. Scale bar, 7 μm.

tion in synaptic FasII, we used two antibodies against FasII to label synaptic boutons: a monoclonal antibody against the intracellular C-terminal tail of FasII (FasIImAb) (Seeger et al., 1993) and an anti-peptide antibody [anti-Fas(ex)] that binds to the extracellular juxtamembrane region of transmembrane FasII. The Fas(ex) antibody binds to surface FasII when applied to fixed but unpermeabilized tissue (Fig. 1*d*) or to living preparations. This antibody was designed to recognize a peptide that is present only on the transmembrane isoforms of FasII and absent from the glycosyl phosphatidyl inositol (GPI)-linked isoform. Accordingly, the antibody recognizes only a single band in Western blots that corresponds to the expected molecular weight of the transmembrane isoforms (see Fig. 4*a*). In contrast, we found that FasIImAb primarily labels submembrane (internal) FasII protein in permeabilized tissue (Fig. 1*e*). It is unclear why FasIImAb does not label most of the surface FasII, but this might be attributable to epitope masking by the binding of the C terminus of surface FasII to the PDZ1–2 domain of Discs-Large (DLG), a cytoplasmic scaffolding protein of the postsynaptic density-95 (PSD-95) family required for synaptic FasII clustering (Thomas et al., 1997). Indeed, DLG and Fas(ex) colocalize exactly (data not shown).

We found that in detergent-permeabilized body wall muscles, internal FasII levels were increased both in homozygous *damph* mutants and in *damph* mutants over *df(2R)vg-C* (Figs. 2*a*, 3*a–f*). In contrast, surface FasII levels were reduced in the mutants (Figs. 2*a*, 3*g–l*). Western blot analysis of body wall muscle extracts, however, did not reveal quantitative differences in total FasII levels between wild-type and *damph* mutants (Fig. 4*a*), demonstrating that in *damph* mutants total FasII levels remain unchanged, and that the differences between wild type and mutants are likely to arise from a decrease in the insertion of cytoplasmic FasII into the membrane, leading to FasII accumulation at the submembrane region.

The above conclusion was further supported by immunocapturing surface FasII *in vivo*. For this experiment, unfixed and unpermeabilized body wall muscle preparations were incubated with anti-Fas(ex) antibody to "capture" surface FasII. Preparations were then homogenized, and the antibody-bound surface FasII was immunoprecipitated with Protein A-coupled Sepharose beads. We found that immunocaptured FasII (representing surface FasII) was substantially decreased in the mutants, whereas FasII in the supernatant (representing internal FasII) was increased (Fig. 4*b*). This result substantiates our findings in the intact preparation, which suggest that Damph is required for normal exocytosis of FasII.

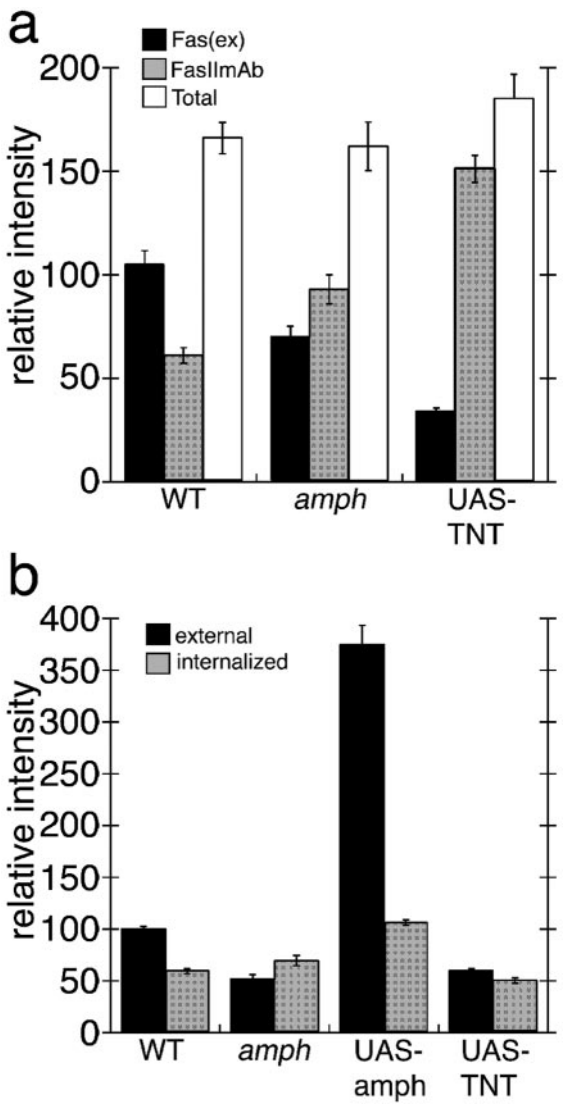

Figure 2. Levels of internal and external FasII are significantly altered in *damph* mutants. The histograms show mean fluorescent intensity measurements across genotypes for Fas(ex) and FasIImAb in fixed preparations (*a*) and external or internalized FasII values obtained in the internalization assay performed on live samples (*b*). *a*, *damph* mutants ($n = 8$) show increased FasIImAb staining ($p < 0.001$) and decreased Fas(ex) staining ($p < 0.001$) compared with wild-type samples ($n = 9$). Staining intensities in UAS-TNT (postsynaptic overexpression of tetanus toxin), as in *damph* mutants, were significantly reduced for Fas(ex) ($p < 0.001$; $n = 13$). *b*, Both *damph* ($n = 6$) and UAS-TNT ($n = 13$) samples show significantly reduced external FasII intensity ($p < 0.001$) while internalizing similar amounts of FasII compared with wild type ($n = 23$). In contrast, UAS-amph samples (postsynaptic overexpression of Damph) ($n = 15$) show a dramatic increase in the level of external FasII ($p < 0.001$) as compared with wild type.

FasII exocytosis is disrupted in *damph* mutants

Additional support for this hypothesis was obtained by studies of FasII internalization *in vivo*. For these experiments, live preparations were incubated with the Fas(ex) antibody to bind surface FasII to saturation. Then, preparations were fixed and labeled

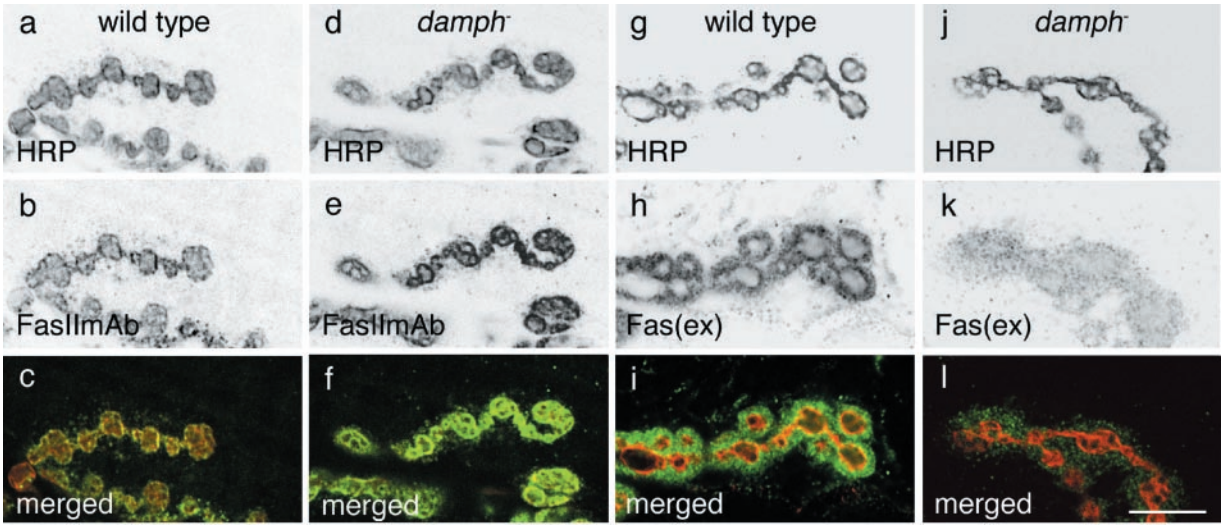

Figure 3. FasII distribution and intensity changes in *damph* mutants. The micrographs show single confocal slices of NMJs from muscles 6 and 7 of third-instar larvae double labeled with anti-FasIImAb (green) and anti-HRP (red) (*a–f*), or anti-Fas(ex) (green) and anti-HRP (red) (*g–l*) in wild-type (*a–c*, *g–i*) and *damph* mutants (*d–f*, *j–l*). Scale bar, 9 μm.

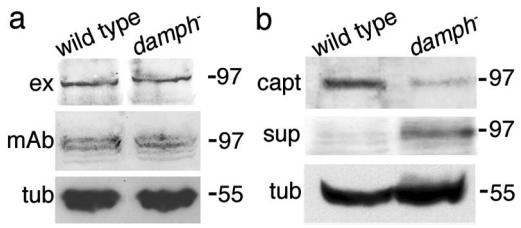

Figure 4. Western blot of body wall muscle extracts and FasII immunocapture. *a*, Western blot of body wall muscle extracts from wild-type and *damph* mutants probed sequentially with the Fas(ex) and FasIImAb antibodies, and with anti-tubulin antibody as a control for equal loading. *b*, Body wall muscle preparations were incubated *in vivo* with the Fas(ex) antibody to immunocapture surface FasII. Then samples were homogenized and surface FasII was immunoprecipitated with protein A-coated Sepharose beads. Immunocaptured FasII (top) and unbound FasII (middle) were visualized by Western blot. The unbound fraction was also probed with an anti-tubulin antibody to control for equal loading. Molecular weights are shown at the right of each blot. Mean densitometric analysis (see Materials and Methods) of Western blot bands in *b* revealed values of 129 for immunocaptured and 26 for the unbound FasII band in wild-type mutants and 52 for immunocaptured and 98 for the unbound FasII band in *damph* mutants. The sums of the values indicating total protein were 155 for wild-type and 150 for *damph* mutants.

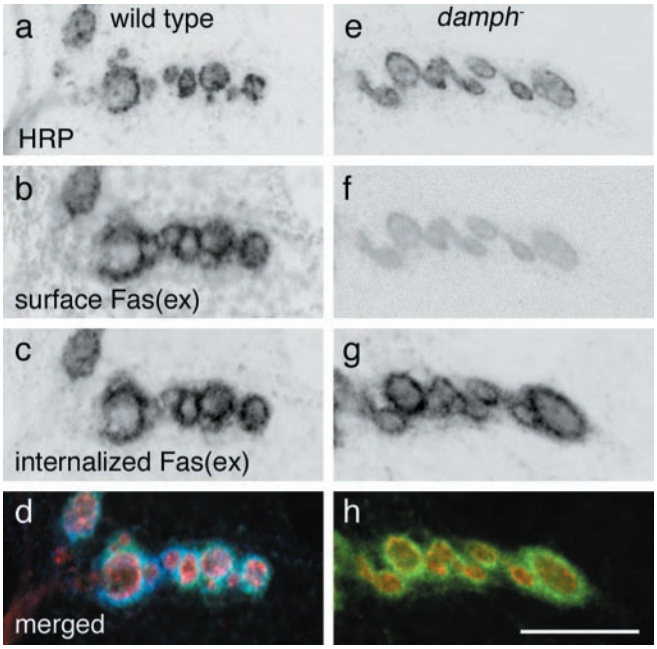

Figure 5. Insertion of FasII into the synaptic membrane is disrupted in *damph* mutants. The micrographs show single confocal slices of NMJs from muscles 6 and 7 of third-instar larvae in wild-type (*a–d*) and *damph* mutants (*e–h*) subjected to an internalization assay (see Materials and Methods). Anti-HRP (red) was used to label the presynaptic membrane (*a*, *e*). Alexa-647 (blue) staining (*b*, *f*) represents FasII molecules bound to the Fas(ex) antibody that remained on or recycled to the cell surface. FITC (green) staining (*c*, *g*) represents FasII molecules bound to the Fas(ex) antibody that have been internalized. In *d* and *h*, the above panels have been merged. Scale bar, 16 μm.

sequentially with two secondary antibodies conjugated to different fluorophores. The initial secondary antibody (Alexa 647 conjugated) was applied in the absence of detergent to label FasII only at the surface. After rinsing and permeabilization, a different secondary antibody (FITC conjugated) was applied to label any anti-Fas(ex)-bound FasII that was internalized during incubation. In wild type, a significant amount of FasII was internalized, but an almost equal amount of FasII remained at the cell surface (Figs. 2*b*, 5*a–d*). In *damph* mutants the amount of internalized FasII was similar to wild type (Figs. 2*b*, 5*g*). In contrast to wild type, however, surface FasII was reduced drastically (Figs. 2*b*, 5*f*). In addition, overexpression of a wild-type *Damph* transgene in the muscle resulted in a dramatic increase in the level of external FasII. Furthermore, the level of internalized FasII after overexpression of wild-type Damph was not reduced but remained high (Fig. 2*b*).

The above observation might result from an artificial alteration of the rate of FasII cycling at the membrane caused by anti-Fas(ex) binding. To rule out this possibility, we examined the levels of FasIImAb immunoreactivity in control samples and in samples treated with anti-Fas(ex) before fixation. We observed no significant differences in the level of FasIImAb immunoreactivity in treated and untreated samples, suggesting that FasII cycling was not altered by Fas(ex) binding.

To determine how fast FasII could be internalized, we performed the antibody incubation in the cold to prevent or slow down internalization. Then, samples were shifted to RT for varying amounts of time (5, 10, 15, and 60 min) to allow membrane

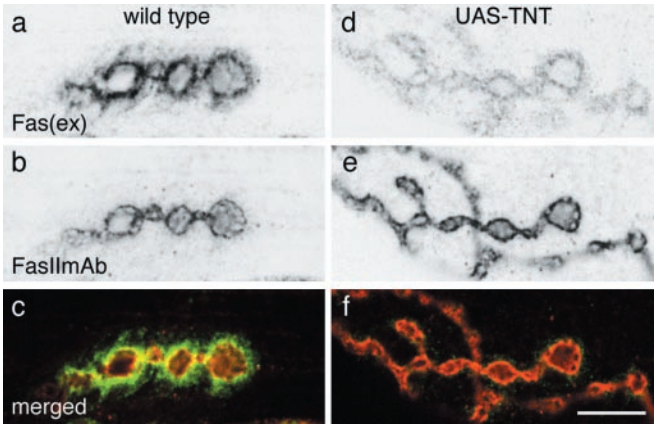

Figure 6. Disruption of SNARE-dependent exocytosis mimics *damph* phenotype. The micrograph shows single confocal slices of NMJs from muscle 6 and 7 of third-instar larvae stained with the Fas(ex) antibody (green) (*a*, *d*) before permeabilization and double labeled with the FasIImAb (red) (*b*, *e*) after permeabilization in wild-type control (*a–c*) and a strain overexpressing UAS-TNT postsynaptically (*d–f*). In *c* and *f*, the above panels have been merged. Scale bar, 7 μm.

protein cycling to resume. We found that an incubation of only 5 min at RT before fixing was enough to reach similar levels of internalized FasII as compared with 2 hr incubations at RT. This suggested that FasII cycling is a relatively fast process, occurring in the order of minutes.

Although the Fas(ex) antibody recognized only the transmembrane FasII isoform, we wondered whether the GPI-linked FasII isoform contributed to transmembrane FasII cycling. This possibility was addressed by eliminating the GPI-linked FasII isoform by incubating the preparations with PI-PLC, a treatment shown to effectively cleave the GPI link (Wright and Copenhaver, 2000). We observed no significant difference in the levels of external or internalized FasII as compared with untreated samples (data not shown).

FasII exocytosis is mediated by a SNARE-dependent mechanism

It has been demonstrated previously that antibody-bound receptors can be recycled to and from the membrane by endocytosis and exocytosis (Passafaro et al., 2001). Therefore, an interpretation of the above results is that in wild-type larvae, FasII is exocytosed and internalized continuously. In *damph* mutants, FasII endocytosis is normal, but exocytosis is impaired. A prediction of this hypothesis is that blocking exocytosis at the postsynaptic cell should mimic the phenotype of *damph* mutants. The basic mechanism of SNARE-dependent exocytosis is conserved in most cell types and across species (Rizo and Sudhof, 2002). Therefore, we tested our prediction by selectively interfering with SNARE function in the postsynaptic cell. For these experiments we expressed tetanus toxin heavy chain (TNT) (Sweeney et al., 1995), which is known to eliminate SNARE function through proteolytic cleavage of vesicle-associated membrane protein synaptobrevin (Schiavo et al., 1992). Furthermore, in separate experiments we interfered with NSF, a protein known to be required for neurotransmitter release (Sollner et al., 1993), by expressing a dominant-negative form of NSF (Stewart et al., 2002). Selective postsynaptic expression of the proteins was achieved by using the UAS/Gal4 system (Brand and Perrimon, 1993), with BG487 as the postsynaptic Gal4 driver (Budnik et al., 1996). We found that expression of TNT in postsynaptic muscles decreased levels of surface FasII in a manner similar to that observed in *damph* mutants (Figs. 2*a*, 6*a*,*d*). There was also a corresponding increase in the intensity of internal FasII staining as observed in the *damph* mutants (Figs. 2*a*, 6*e*). In contrast, no change in the levels of surface or internal FasII was observed by postsynaptic expression of an inactive TNT form (IMPTNT) (Sweeney et al., 1995). Similarly, *in vivo* FasII internalization assays performed in the strain overexpressing TNT postsynaptically showed cycling defects similar to those observed in *damph* mutants. The same outcome, a phenocopy of the *damph* mutant phenotype, was obtained by postsynaptic expression of the dominant-negative NSF (data not shown). Together, these results are consistent with the idea that Fas II is exocytosed through a SNARE-dependent mechanism and that Damph might be involved in this process.

Discussion

Studies in vertebrates have suggested two roles for Amph: endocytosis of synaptic vesicles and deformation of membranes required for the generation of muscle T-tubules. Although this second function appears to be conserved in *Drosophila* (Razzaq et al., 2001), *damph* mutants show no defects in presynaptic vesicle cycling. Moreover, *damph* mutants show no genetic interaction with *Shibire*, the gene encoding the *Drosophila* Dynamin homolog (Zelhof et al., 2001), suggesting that in the fly, Damph does not play a role in endocytosis (Leventis et al., 2001; Razzaq et al., 2001; Zelhof et al., 2001).

At the *Drosophila* larval NMJ, Damph is enriched at the postsynaptic region (Leventis et al., 2001; Razzaq et al., 2001; Zelhof et al., 2001), suggesting a role in postsynaptic function. The observations that Damph colocalizes with FasII at the postsynaptic membrane, and that both severe hypomorphic *fasII* and null *damph* mutants show a similar decrease in synaptic bouton number at the NMJ, prompted us to examine more closely the relationship between the two proteins. Total FasII levels were similar in both wild-type and *damph* mutants as determined in Western blots; however, the amount of FasII integrated into the synaptic membrane, revealed by labeling the extracellular region of FasII in unpermeabilized tissue, was reduced. This observation suggested that in *damph* mutants, FasII cycling at the membrane, in particular the insertion (exocytosis) of FasII into the plasma membrane, might be impaired. Compelling evidence supporting this model was obtained from *in vivo* studies of FasII internalization. In wild type, antibody-bound FasII complexes underwent cycling by continuous internalization and subsequent reinsertion into the membrane. In contrast, in *damph* mutants, although internalization appeared normal, the reinsertion of the complexes into the plasma membrane was strikingly decreased. A potential concern in the *in vivo* antibody binding experiments is that Fas(ex) binding could artificially induce or accelerate FasII endocytosis, which would manifest in a change in internal levels of FasII. We did not detect any change in the levels of internalized FasII when samples were treated with the Fas(ex) antibody before staining with FasIImAb; however, the possibility that both internalization and exocytosis are affected simultaneously cannot be excluded by these experiments.

On the basis of our results, we favor the hypothesis that Damph is involved in FasII exocytosis; however, a counter hypothesis is that Damph plays a role in the negative regulation of endocytosis. Although we cannot rule out this possibility completely, our observation that postsynaptic overexpression of wild-type Damph dramatically increases levels of external FasII without a decrease in internalization supports a role in exocytosis. Negative regulation of endocytosis would be expected to increase

levels of surface FasII and decrease internalization. The higher than normal levels of internalized FasII observed after overexpression of wild-type Damph is likely to be caused by an enhanced pool of surface FasII that becomes labeled by the Fas(ex) antibody in this mutant and results in an increase in the number of FasII-antibody complexes undergoing internalization in our assay. Interestingly, FasII exocytosis appeared to use the same machinery as required for exocytosis of synaptic vesicles, because interfering with SNAREs mimicked *damph* mutant phenotypes. The tetanus toxin construct has been used in a genetic approach to study the role of different neurons in several forms of behavior such as olfaction, mechanoreception, vision, and learning and memory (Martin et al., 2002). SNARE-dependent mechanisms are conserved through species and have also been observed in postsynaptic cells of vertebrates (Lledo et al., 1998; Braithwaite et al., 2002). Therefore, it is not surprising to observe an exocytosis type of effect in larval muscle cells after overexpression of UAS-TNT or a dominant-negative NSF.

Because of the structural similarities between invertebrate and vertebrate forms of Amph, it was surprising to find that in each system Amph performs seemingly opposite functions: exocytosis in invertebrates and endocytosis in vertebrates. Although both the fission and fusion of a vesicle require different subsets of proteins, they both also involve common processes, in particular the requirement to deform membrane. Such a role for Amph in membrane deformation has been supported by studies with liposomes, in which addition of purified Amph resulted in membrane evagination, forming tubular structures (Takei et al., 1999). This ability of the protein could account for its role in both exocytosis and endocytosis. It will be interesting, from an evolutionary point of view, to determine whether the role of Amph in exocytosis is applicable in other tissues, particularly at mammalian postsynaptic cells.

At vertebrate central synapses, postsynaptic exocytosis of AMPA-type glutamate receptors (Passafaro et al., 2001), also accomplished by a SNARE-dependent mechanism (Lledo et al., 1998; Braithwaite et al., 2002), is central to regulation of synapse strength during plasticity (Luscher et al., 1999). The precise mechanism of the activity-dependent insertion of glutamate receptors and cell adhesion molecules into the membrane still remains incompletely understood. At the fly NMJ, FasII is crucial for synapse stability, but a local decrease in FasII levels is necessary for the generation of new synaptic boutons (Schuster et al., 1996a,b; Zito et al., 1999). These dynamic changes in FasII localization are likely to be mediated by activity-dependent regulation of synaptic clustering of FasII by the PSD-95 family member DLG (Thomas et al., 1997; Koh et al., 1999), and by MAP kinase pathway-dependent FasII downregulation (Koh et al., 2002). We suggest that these mechanisms are complemented by SNARE-dependent FasII exocytosis. In wild type, about half of type I boutons (~40 boutons) are formed during the last day of larval development, suggesting that the generation of each new synaptic bouton during NMJ expansion takes ~2 hr (Gorczyca et al., 1993). Both this process and the regulation of synaptic levels of FasII are regulated by activity (Schuster et al., 1996b; Koh et al., 1999). Our observations of the time course of FasII internalization suggest that FasII is internalized as fast as within 5 min. This relatively fast cycling of FasII at mature larval synapses would be compatible with a potential activity-dependent regulation of postsynaptic FasII by Damph.

In conclusion, our studies suggest a novel role for Damph in vesicle exocytosis, specifically in SNARE-dependent postsynaptic exocytosis of FasII. A key to further understanding the finer details of activity-dependent protein trafficking would be to resolve the exact step in the exocytotic process that is regulated by Damph and to investigate whether its function extends to other molecules such as neurotransmitter receptors and ion channels. The simple experimental system described here could also be used to further understand dynamic regulation of protein trafficking at the postsynapse during synaptic plasticity.

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