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A Presynaptic Homeostatic Signaling System Composed of the Eph Receptor, Ephexin, Cdc42 and Ca_v2.1 Calcium Channels

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Abstract

Homeostatic signaling systems have been implicated in the modulation of presynaptic neurotransmitter release, but the underlying mechanisms remain unknown. In a screen that was performed blind to gene identity we isolated mutations in *Drosophila ephexin* (Rho-type guanine nucleotide exchange factor) that disrupt the homeostatic enhancement of presynaptic release following impairment of postsynaptic glutamate receptor function at the *Drosophila* NMJ. We show that Ephexin is sufficient presynaptically for synaptic homeostasis and localizes in puncta throughout the nerve terminal. However, *ephexin* mutations do not alter other aspects of neuromuscular development including morphology or active zone number. We then show that, during synaptic homeostasis, Ephexin functions primarily with Cdc42 in a signaling system that converges upon the presynaptic Ca_v2.1 calcium channel. Finally, we show that Ephexin binds the *Drosophila* Eph receptor (Eph) and *Eph* mutants disrupt synaptic homeostasis. Based on these data we propose that Ephexin/Cdc42 couples synaptic Eph signaling to the modulation of presynaptic Ca_v2.1 channels during the homeostatic enhancement of presynaptic release.

INTRODUCTION

Homeostatic signaling systems, operating at the level of individual nerve and muscle cells, are believed to interface with the mechanisms of neural plasticity to ensure that neural function remains stable over time (Burrone and Murthy, 2003; Davis, 2006; Davis and Bezprozvanny, 2001; Frank et al., 2006; Marder and Goaillard, 2006; Marder and Prinz, 2002; Perez-Otano and Ehlers, 2005; Turrigiano and Nelson, 2004). There are two general mechanisms by which homeostatic regulatory systems have been proposed to control neuronal excitability. In one paradigm, chronic perturbations of neuronal activity have been observed to cause compensatory changes to ion channel or neurotransmitter receptor abundance (Davis, 2006; Marder and Prinz, 2002; Turrigiano and Nelson, 2004). In a second paradigm, the homeostatic modulation of synaptic transmission can be expressed as a change in presynaptic vesicle release, observed at both central (Murthy et al., 2001; Thiagarajan et al., 2005) and peripheral synapses (Davis, 2006). The most well characterized example of this type of homeostatic signaling is observed at the neuromuscular junction (Davis, 2006; Davis et al., 1998; Frank et al., 2006; Petersen et al., 1997). For example, impaired postsynaptic neurotransmitter receptor

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function at the *Drosophila* NMJ initiates a rapid, compensatory increase in presynaptic release that offsets impaired receptor function and restores muscle excitation (Davis, 2006; Frank et al., 2006). This homeostatic signaling system can be initiated by genetic mutation of the glutamate receptor subunit *GluRIIA* (Petersen et al., 1997), genetic alteration of glutamate receptor function (Davis et al., 1998), acute pharmacological inhibition of glutamate receptor function (Frank et al., 2006) and perturbation of muscle membrane excitability (Frank et al., 2006; Paradis et al., 2001).

Work at the *Drosophila* NMJ has begun to uncover genes that are required for homeostatic compensation. It was recently demonstrated that homeostatic modulation of presynaptic release (synaptic homeostasis) is blocked by mutations that disrupt the function of a presynaptic $Ca_v2.1$ calcium channel subunit encoded by the gene *cacophony* (*cac*) (Frank et al., 2006). The observation that synaptic homeostasis, following inhibition of postsynaptic glutamate receptors, is blocked by mutations in a presynaptic calcium channel has been taken as further molecular evidence for the existence of a retrograde signal at this NMJ (Davis, 2006; Frank et al., 2006). The nature of this homeostatic, retrograde signal has been of great interest. Recent data have provided evidence that Bone Morphogenic Protein (BMP) signaling can function as a retrograde signal at the *Drosophila* NMJ, coupling the developmental growth of the muscle to the growth of the presynaptic nerve terminal (Aberle et al., 2002; McCabe et al., 2003). Although this same BMP signaling system is required for synaptic homeostasis at the *Drosophila* NMJ, the BMPs do not function as the retrograde signal that directly modulates presynaptic neurotransmitter release (Goold and Davis, 2007). Rather, the BMPs confer competence upon the motoneurons such that they are able to express homeostatic plasticity (Goold and Davis, 2007). Thus, the nature of the retrograde signal that directly mediates the homeostatic modulation of presynaptic release remains unknown. Likewise, nothing is known regarding the signaling systems that reside within the presynaptic nerve terminal that couple retrograde signaling to the modulation of $Ca_v2.1$ calcium channels that appears necessary for a homeostatic change in synaptic vesicle release.

Here we identify and characterize core components of a presynaptic signaling system that is necessary for the homeostatic modulation of presynaptic release at the *Drosophila* NMJ. We provide evidence that Ephexin (Exn), a Rho-type guanine nucleotide exchange factor (Rho-GEF), is a presynaptic protein that is required for the homeostatic modulation of vesicle release. We then provide evidence that, during synaptic homeostasis, Exn acts primarily via Cdc42 within a signaling system that converges upon the $Ca_v2.1$ calcium channel. Finally, we show that *Drosophila* Ephexin, like its vertebrate homologue, binds to the Eph receptor. We then show that *Eph* mutants disrupt the homeostatic modulation of presynaptic release at the *Drosophila* NMJ. Therefore, we hypothesize that the *Drosophila* Eph receptor may receive a retrograde signal at the presynaptic nerve terminal. Exn and Cdc42 could then couple Eph activation to the modulation of $Ca_v2.1$ calcium channels necessary for the homeostatic modulation of presynaptic release. These data not only significantly advance our understanding of the presynaptic signaling systems that mediate a homeostatic modulation of presynaptic release, but also define a function for Rho-GTPase signaling in the modulation of synaptic transmission at a fast, glutamatergic synapse.

RESULTS

As part of an ongoing, large-scale screen, we have identified mutations in the gene *CG3799* that disrupt the homeostatic modulation of presynaptic neurotransmitter release at the *Drosophila* NMJ. The gene *CG3799* is annotated as the *Drosophila* homologue of the vertebrate Rho-type guanine nucleotide exchange factor (Rho-GEF) termed *exn*. The N-terminal half of *CG3799* is not conserved. However, the C-terminal half is highly homologous to vertebrate *exn*, including the presence of a Dbl Homology-Plextrin Homology (DH-PH) domain (Rho-

GEF domain) and a SH3 domain (Shamah et al., 2001; Sahin et al., 2005). These homologies strongly predict that *CG3799* functions as a Rho-GEF. Based upon sequence homology, domain organization and the current annotation of this gene in FlyBase, we refer to this gene as *Drosophila exn* (Figure 1).

To complete a genetic analysis of *Drosophila exn* we took advantage of a P-element insertion mutation in the *exn* gene (*exn^{EY01953}*; BDGP gene disruption project) (Bellen et al., 2004). This transposon insertion resides within the coding sequence of exon 2 (Figure 1A) and, as such, is predicted to cause a severe loss of gene function. In order to perform a genetic analysis of *Drosophila exn* we generated additional alleles. We first excised the P(*EY01953*) transposon and screened for both imprecise and precise P-element excisions. We isolated two imprecise excisions of the P(*EY01953*) transposon termed *exn^{EY-Δ50}* and *exn^{EY-Δ23}*. These new mutations are deletions that remove large portions of the *exn* gene and are predicted to be null mutations (Figure 1). We also isolated *exn^{EY-5}*, which is a precise excision of the P(*EY01953*) transposon (Figure 1A). We used these genetic tools to further test the requirement of *exn* for the homeostatic modulation of presynaptic release at the *Drosophila* NMJ.

***exn* mutations disrupt synaptic homeostasis**

Mutations in the *GluRIIA* subunit of the muscle-specific glutamate receptor (*GluRIIA^{SP16}*) cause a significant decrease in the average spontaneous miniature release event (mepsp) amplitude (Petersen et al., 1997). The decrease in mepsp amplitude is offset by a significant increase in presynaptic transmitter release as assessed by calculation of quantal content (see Methods). This increase in quantal content has been confirmed to represent a change in presynaptic transmitter release by analysis of failures (Petersen et al., 1997) and analysis of short-term synaptic plasticity (Frank et al., 2006). This effect has been interpreted as evidence for a homeostatic modulation of presynaptic transmitter release, a conclusion supported by additional electrophysiological experiments using pharmacological agents to impair postsynaptic glutamate receptor function (Frank et al., 2006).

To analyze the requirements of *exn* for synaptic homeostasis we generated double mutants of all *exn* alleles with the glutamate receptor mutation, *GluRIIA^{SP16}*. We first demonstrate that spontaneous miniature release event amplitude is significantly decreased in the *GluRIIA^{SP16}; exn^{EY01953}* double mutant, an effect that is similar to that in the *GluRIIA^{SP16}* mutant alone (Figure 2A). However, the homeostatic increase in presynaptic transmitter release is severely impaired in these double mutant animals (Figure 2; $p=0.01$). These data suggest that disruption of the *exn* gene prevents the normal expression of synaptic homeostasis at the *Drosophila* NMJ. To confirm the specificity of this effect, we examined animals in which we place the precise P-element excision allele (*exn^{EY-5}*) in the background of the *GluRIIA^{SP16}* mutation. In these animals, the significant decrease in mepsp amplitude is offset by an increase in presynaptic release, restoring EPSP amplitudes toward control values, thereby demonstrating normal homeostatic modulation of presynaptic transmitter release (Figure 2A).

Presynaptic Ephexin is sufficient to rescue synaptic homeostasis

The *exn* gene appears to be ubiquitously expressed throughout most tissues in the *Drosophila* embryo including the central and peripheral nervous systems, as assessed by RNA *in situ* hybridization (data not shown). Therefore, we performed genetic rescue experiments to determine if *exn* functions in the presynaptic neuron during the homeostatic modulation of presynaptic release. To do so, we generated transgenic animals that allow the expression of a *UAS-YFP-exn* transgene (YFP is placed at the extreme N-terminus of the *exn* cDNA, see methods). We then expressed *UAS-YFP-exn* using a pan-neuronal GAL4 driver (*elav-GAL4*) in the *GluRIIA; exn* double mutant background. We performed the rescue experiments using both the *exn* P-element insertion mutation *exn^{EY01953}* and the putative null allele *exn^{EY-Δ23}* in

the *GluRIIA* mutant background. We find that presynaptic expression of *UAS-YFP-exn* is sufficient to fully rescue the expression of synaptic homeostasis in both the *GluRIIA; exn^{EY10953}* and the *GluRIIA; exn^{EY-Δ23}* double mutant backgrounds (Figure 3). These experiments demonstrate that presynaptic expression of *exn* is sufficient to restore synaptic homeostasis in an *exn* mutant background.

To visualize Exn localization within the presynaptic nerve terminal, we imaged YFP-Exn in animals that express *UAS-YFP-exn* using the *elav-GAL4* driver. Since presynaptic expression of YFP-Exn is sufficient to rescue defective synaptic homeostasis in the *exn* mutant, the distribution of ectopically expressed YFP-Exn should reflect, at least in part, the endogenous localization of Exn during the process of synaptic homeostasis. In support of this approach, numerous synaptic proteins have been shown to have appropriate sub-synaptic localization when overexpressed epitope-tagged proteins are visualized (Heerssen et al., 2008; Marek and Davis, 2002; Sweeney and Davis, 2002).

We find that YFP-Exn is efficiently trafficked to the presynaptic nerve terminal at the NMJ (Figure 4). YFP-Exn localizes to small puncta that are distributed throughout the presynaptic nerve terminal. These YFP-Exn puncta are found in close proximity to active zones, defined by co-staining with antibodies that recognize postsynaptic glutamate receptor clusters (Figure 4B, C). However, our image analysis indicates that YFP-Exn is not strictly an active zone associated protein since it is not found in direct opposition to all glutamate receptor clusters, whereas the active zone associated protein Bruchpilot (Brp) is found opposed to each postsynaptic glutamate receptor cluster (Pielage et al., 2006; Wagh et al., 2006). From these data, we conclude that Exn is trafficked to the presynaptic nerve terminal and resides in a position where it could reasonably participate in the modulation of presynaptic vesicle release.

Calcium cooperativity of neurotransmitter release is normal in *exn* mutants

We next examined baseline synaptic transmission in the *exn* mutant background. We observe a small, but statistically significant, defect in synaptic vesicle release in the *exn* mutants compared to wild type controls (Figure 5A, B). We also observe a trend toward an increase in mepsp amplitude ($p > 0.10$). We assayed presynaptic release over a range of external calcium concentrations (0.2 to 0.5mM). We find a statistically significant decrease in presynaptic release in *exn* compared to wild type at each calcium concentration tested (Figure 5C). Nevertheless, our data indicate that the calcium cooperativity of vesicle release remains normal in the *exn* mutant background. These data highlight a requirement for *exn* within the presynaptic nerve terminal, either during NMJ development, which could have a basal requirement for synaptic homeostasis, or in the regulation of synaptic vesicle release. Since synaptic homeostasis is normal in mutant backgrounds that disrupt baseline synaptic transmission to a far greater extent than *exn* (including mutations in *syx1A* and *cysteine string protein*; Goold and Davis, 2007), we speculate that the change in baseline synaptic transmission is not the primary cause of impaired synaptic homeostasis, though it could contribute. We also find that the percent decrease in quantal content in *exn* compared to wild type is significantly less than the percent decrease in quantal content in *GluRIIA; exn* double mutant animals compared to *GluRIIA* mutants (Figure 2; $p < 0.05$). Again, these data suggest that Exn has an important function during synaptic homeostasis.

Normal number of active zones in *exn* mutants

We next determined whether *exn* mutations alter anatomical NMJ development or active zone number. It has been established that synaptic growth is normal in the *GluRIIA^{SP16}* mutant background (Petersen et al., 1997), and that the rapid induction of synaptic homeostasis is protein synthesis independent and occurs without a change in NMJ morphology or active zone number (Frank et al., 2006). However, it remains possible that a defect in NMJ development

could disrupt the NMJ such that it could not express homeostatic plasticity. Therefore, we examined NMJ morphology and active zone number in *GluRIIA^{SP16}* animals compared to the *GluRIIA^{SP16}; exn* double mutant animals. We stained NMJs with anti-HRP and anti-Bruchpilot (Brp) antibodies, which mark the presynaptic membrane and presynaptic active zone, respectively (Pielage et al., 2006; Wagh et al., 2006). We find that the *GluRIIA^{SP16}; exn* double mutants have a slight, though statistically significant, increase in bouton number (Figure 6). Thus, decreased NMJ growth cannot account for impaired synaptic homeostasis in these animals. We also quantified the average number of active zones per NMJ using anti-Brp to define active zones as done previously (Pielage et al., 2006). Active zones were visualized and quantified in three-dimensions, allowing us to quantify total active zone number per NMJ. We find no difference in average active zone number per NMJ in *GluRIIA* mutants versus *GluRIIA^{SP16}; exn* double mutants (Figure 6F). Thus, the *exn*-dependent block of synaptic homeostasis is not a secondary consequence of defective morphological development or a deficit in presynaptic active zone number. It is formally possible that the small increase in bouton number reflects a response of the NMJ to impaired synaptic homeostasis. However, this seems unlikely because the change is small and there is no change in total active zone number.

Exn-dependent signaling converges upon presynaptic Ca_v2.1 calcium channels to achieve homeostatic modulation of presynaptic release

In a previous study, we demonstrated that *cacophony* (*cac*) mutations, which impair the pore-forming subunit of the Ca_v2.1 presynaptic calcium channel, completely block synaptic homeostasis at the Drosophila NMJ (Frank et al., 2006). To test for a genetic interaction between *exn* and *cac*, we generated double heterozygous mutant combinations (*cac/+; exn/+*) within a *GluRIIA* mutant background. The *exn/+* heterozygous mutations (*exn^{EY01953/+}* and *exn^{EY-423/+}*) alone had no significant effect on synaptic homeostasis when placed in the *GluRIIA* mutant background (Figure 7A; $p > 0.2$; and data not shown), while the presence of the *cac^{S/+}* mutation (a point mutation) in the *GluRIIA* mutant background showed a mild suppression (24% decrease; $p < 0.05$) of homeostatic compensation (Figure 7A). However, the presence of both heterozygous mutations in the *GluRIIA* mutant (*cac^{S/+}; GluRIIA; exn/+*) caused a complete block of homeostatic compensation (Figure 7A). A quantitatively similar genetic interaction is observed between a *cac* null mutation (*cac^{HC129}*) and *exn/+*. In these experiments the presence of the heterozygous *cac^{HC129/+}* mutation had little effect on quantal content in the *GluRIIA* mutant background ($p > 0.05$), but blocked synaptic homeostasis when combined with the heterozygous *exn/+* mutation in the *GluRIIA* mutant ($p < 0.01$ when compared with either *GluRIIA; exn/+* or *cac^{HC129/+}; GluRIIA*; data not shown). A strong genetic interaction between heterozygous mutations, such as observed here, can be taken as evidence that the two genes function within the same biological process, acting either within the same signaling system or in parallel signaling pathways.

To further examine the relationship between Exn and Cac in this homeostatic signaling system, we asked whether expression of *cac* (*UAS-GFP-cac*) in the *GluRIIA; exn* double mutant animals could rescue the defect in synaptic homeostasis. The *UAS-GFP-cac* channel is expressed, localizes to presynaptic active zones (Wagh et al., 2006) and is sufficient to rescue viability in the *cac* null mutation demonstrating that this channel incorporates into presynaptic active zones. As a control, we first verified that expression of the *UAS-GFP-cac* cDNA alone has no effect on baseline synaptic transmission (data not shown), confirming previously published data (Kawasaki et al., 2002). Next we demonstrate that synaptic homeostasis is significantly restored when *UAS-GFP-cac* is expressed in the *exn; GluRIIA* double mutant background (Figure 7B, C; $p < 0.01$). Since expression of *UAS-GFP-cac* alone has no effect on baseline transmission, the partial rescue of synaptic homeostasis cannot be attributed to a non-specific potentiation of release. Rather, we conclude that Cac over-expression compensates for

a specific defect caused by the *exn* mutation that is responsible for the block of synaptic homeostasis in a *GluRIIA* mutant background. These genetic data are consistent with a model in which the *Cac* channel resides downstream of *Exn* in a presynaptic, homeostatic signaling system at the *Drosophila* NMJ.

Evidence for altered presynaptic calcium channel activity in the *GluRIIA* mutant background

To this point our genetic data support the conclusion that *exn* is required upstream of the *cac* channel during synaptic homeostasis, but the data do not address whether modulation of the presynaptic calcium channel normally occurs in a *GluRIIA* mutant background. Therefore, we turned to the use of Cadmium (Cd^{2+}) and Nickel (Ni^{2+}), which are described as indiscriminant calcium channel antagonists (Mintz et al., 1995). In cultured *Drosophila* neurons, both divalents block the $\text{Ca}_v2.1$ calcium current (Peng and Wu, 2007). Here we demonstrate that presynaptic release (quantal content) in the *GluRIIA* mutant background is significantly more sensitive to extracellular application of Cd^{2+} ($3\mu\text{M}$) than is the wild type NMJ (Supplemental Figure 2A). There is an even greater disparity between wild type and *GluRIIA* when using Ni^{2+} (0.5mM) instead of Cd^{2+} (Supplemental Figure 2A). Although both Cd^{2+} and Ni^{2+} reduce mepsp amplitude (Supplemental Figure 2B), the percent change in mepsp amplitude cannot account for increased sensitivity of presynaptic release to both Cd^{2+} and Ni^{2+} observed in the *GluRIIA* mutant. Finally, there is no effect of these divalents on resting membrane potential (Supplemental Figure 2C). The differential effects of Cd^{2+} and Ni^{2+} are consistent with the conclusion that synaptic homeostasis in the *GluRIIA* mutant is achieved, at least in part, through an effect on presynaptic calcium channels that could include a change in calcium channel number, activity or localization. This conclusion is also consistent with our previously published observation that $\text{Ca}_v2.1$ channel mutations block synaptic homeostasis in the *GluRIIA* mutant (Frank et al., 2006). When taken together with our genetic analyses described above, these data support a model in which *Exn*-dependent signaling converges upon the presynaptic $\text{Ca}_v2.1$ calcium channel to achieve a homeostatic modulation of presynaptic release.

Ephexin signaling during the rapid induction of synaptic homeostasis

We recently demonstrated that application of a sub-blocking concentration of the glutamate receptor antagonist philanthoxin (PhTx) can induce a compensatory, homeostatic increase in presynaptic release in approximately 10 minutes (Frank et al., 2006). Thus, application of PhTx can be used to probe the mechanisms responsible for the rapid induction of homeostatic signaling, in contrast to assaying homeostatic compensation in a *GluRIIA* mutant animal, which reveals the effects of persistent homeostatic signaling over a period of 3–4 days. Several mutations, including alleles of *cac* block both PhTx-dependent and *GluRIIA*-dependent synaptic homeostasis, indicating that the rapid induction and sustained expression of synaptic homeostasis share molecular mechanisms (Frank et al., 2006; Goold and Davis, 2007). Therefore, we applied PhTx ($4\mu\text{M}$) to the *exn* mutant as done previously (Frank et al., 2006; Goold and Davis, 2007). To our surprise we find that the PhTx-dependent, rapid induction of synaptic homeostasis is normal in the *exn* mutant (Supplemental Figure 1). These data suggest that *exn* may be dispensable for the rapid induction of synaptic homeostasis. To further examine the role of *Exn*-dependent signaling during the rapid induction of synaptic homeostasis, we tested the *cac/+; exn/+* double heterozygous mutant animals that we show disrupt synaptic homeostasis in the *GluRIIA* mutant background (see above). Each heterozygous mutant alone has no effect on the rapid induction of homeostatic signaling following PhTx application. However, the double heterozygous mutant animals show a severe disruption of synaptic homeostasis (Supplemental Figure 1). Quantitatively identical results were obtained when using two independent *cac* alleles in this experiment. Thus, although *exn* mutations alone do not block the rapid induction of synaptic homeostasis, we provide evidence that the larger signaling system that includes both *exn* and *cac* is required for both the rapid induction and

sustained expression of synaptic homeostasis. Why, then, does *exn* have a greater effect on synaptic homeostasis in the *GluRIIA* mutant background? The *GluRIIA* mutation is a persistent homeostatic stress and might place an increased burden on the full functionality of the homeostatic signaling system. Alternatively, Exn signaling may have a more pronounced role during the sustained expression of synaptic homeostasis. To date, the molecular mechanisms that convert the rapid induction of synaptic homeostasis into the persistent expression of synaptic homeostasis remain unknown.

Evidence that Exn acts primarily via Cdc42 during synaptic homeostasis

The Exn RhoGEF domain is conserved in *Drosophila*. Therefore, we sought to determine which Rho-type GTPase(s) might function with Exn during the mechanisms of synaptic homeostasis. The *Drosophila* homolog of RhoA is called Rho1. There exist several published loss-of-function *Rho1* alleles, including *Rho1⁷²⁰*, which is a P-element transposon excision allele that removes the translational start site of Rho1 (Strutt et al., 1997). A second allele that we used is *Rho1^{k02107b}*, which is an intronic P-element transposon insertion (Magie et al., 1999). Both homozygous mutations are embryonic lethal (Magie et al., 1999; Strutt et al., 1997). To test for defects in synaptic homeostasis, we generated animals with a *Rho1/+* heterozygous mutation in a *GluRIIA* mutant background. We find that the *Rho1^{720/+}* heterozygotes and the *Rho1^{k02107b/+}* heterozygotes both cause a partial, but statistically significant ($p \leq 0.05$), suppression of synaptic homeostasis (Figure 8B). We next tested whether *Rho1* and *exn* interact genetically by generating transheterozygous *Rho1/+*, *exn/+* animals in a *GluRIIA* mutant background. However, we did not observe any enhancement of the *Rho1/+* mutant phenotype in these animals. These data suggest that loss of Rho1 influences the expression of synaptic homeostasis, but Rho1 is not the primary GTPase involved in Exn signaling during synaptic homeostasis.

We conducted similar experiments with *Rac1* and *Cdc42* mutations. For *Rac1*, we used the *Rac1^{J11}* allele, a null missense mutation (G60E) that alters a glycine that is ubiquitous in the GTPase superfamily (Hakeda-Suzuki et al., 2002; Ng et al., 2002). As with *Rho1/+* heterozygotes, the *Rac1^{J11/+}* heterozygous mutation causes a mild suppression of synaptic homeostasis when placed in the *GluRIIA* mutant background (Figure 8A). Again, the *Rac1/+* mutant showed no further effect on synaptic homeostasis when combined with a heterozygous *exn/+* mutation (Figure 8A). Indeed, this double heterozygous mutant combination in the *GluRIIA* background shows normal homeostatic compensation, underscoring that Rac1 is unlikely to be the GTPase that functions with Exn during synaptic homeostasis.

Next, we tested a role for *Cdc42*. We examined three independent loss-of-function alleles, *Cdc42²*, *Cdc42³* and *Cdc42⁴* (Fehon et al., 1997; Genova et al., 2000). *Cdc42³* is an embryonic lethal missense mutation, G114D; *Cdc42⁴* is an embryonic lethal splice acceptor alteration; and *Cdc42²* alters a splice donor sequence (Fehon et al., 1997; Genova et al., 2000). Synaptic homeostasis in the *GluRIIA* mutant was unaffected by the presence of a *Cdc42^{3/+}* or *Cdc42^{4/+}* heterozygous mutation (Figure 8C). However, in this case, the *Cdc42* alleles showed a strong genetic interaction with *exn*. Synaptic homeostatic signaling was completely blocked in animals that are double heterozygous for *Cdc42/+*; *exn/+* in the *GluRIIA* mutant background (Figure 8C). We extended this analysis to the third *Cdc42* allele (*Cdc42²*) with quantitatively similar results (data not shown). Finally, having implicated *Cdc42* in the mechanisms of synaptic homeostasis we controlled for effects on synapse morphology. There is no deficit in synaptic bouton number in *Cdc42^{3/+}*; *GluRIIA* or *Cdc42^{4/+}*; *GluRIIA* or *Cdc42^{3/+}*; *GluRIIA*; *exn/+* compared to wild type or *GluRIIA* controls (data not shown; $p > 0.1$, $N > 10$ for all genotypes). Together, these data indicate that *Cdc42* is the primary small GTPase that functions with Exn during the homeostatic modulation of presynaptic release.

Finally, since *exn* shows a strong genetic interaction with mutations in the $\text{Ca}_v2.1$ channel (*cac/+*), we asked whether mutations in *Rac1*, *Rho1* and *Cdc42* might show a genetic interaction with mutations in *cac* as well. To our surprise, although *Cdc42* is the only GTPase to show a strong genetic interaction with *exn*, heterozygous mutations in all three GTPases block synaptic homeostasis when combined with a heterozygous *cac/+* mutation in the *GluRIIA* mutant background (Figure 8). From these data, we conclude that loss of a single copy of *cac* has rendered the presynaptic nerve terminal highly sensitive to mutations in *Rho1*, *Rac1* and *Cdc42* during synaptic homeostasis. It is possible that the heterozygous *cac* mutation has rendered the presynaptic terminal sensitive to any mutation that impairs, even modestly, synaptic homeostasis. Consistent with such a possibility, the *Rho1/+* and *Rac1/+* heterozygous mutations cause a mild suppression of synaptic homeostasis and show a strong genetic interaction with *cac* (Figure 8).

The Ephrin receptor binds Exn and is required for synaptic homeostasis

Exn was originally identified as an Eph-receptor interacting protein that can convey signaling from the EphA receptor to the actin cytoskeleton through activation of the small GTPases RhoA, Rac and Cdc42 (Shamah et al., 2001; Sahin et al., 2005). In contrast to vertebrates, the *Drosophila* genome encodes only a single Eph receptor that shows equal similarity to both the A and B classes of Eph receptors (Dearborn et al., 2002; Scully et al., 1999). During development, the Eph receptor is highly expressed throughout the nervous system and is targeted to axons (Boyle et al., 2006; Scully et al., 1999). Therefore, the Eph receptor is present within developing neurons and is a prime candidate to receive a trans-synaptic signal involved in synaptic homeostasis.

First, we tested whether the interaction between *Drosophila* Exn and the Eph receptor is conserved. Using a yeast-2-hybrid approach we demonstrate that the kinase-domain of the Eph receptor binds to the C-terminal Rho-GEF domain of Exn (Figure 9). We also observe an interaction between the Eph kinase domain and the SH3 domain of Exn (Figure 9). These data are similar to the interaction observed in vertebrates, though an interaction mediated by the SH3 domain of Exn was not previously observed in vertebrate homologues (Shamah et al., 2001; Sahin et al., 2005).

These data suggest that Exn could function downstream of the Eph receptor at the presynaptic nerve terminal of the *Drosophila* NMJ. To test a potential requirement of the *Drosophila* Eph receptor in synaptic homeostasis, we analyzed a previously published, molecularly characterized Eph receptor deletion mutation, *Eph^{x652}* (Boyle et al., 2006) by electrophysiology. First, we demonstrate that *Eph^{x652}* mutants have a slight, but significant ($p < 0.05$), defect in quantal content and EPSP amplitude (Figure 9D,E). However, there is no significant difference in average mepsp amplitude (Figure 9C). The calculation of quantal content (average EPSP/average mepsp) could be influenced by the trend toward larger mepsp amplitudes ($p > 0.1$; Figure 9C) combined with the slight decrease in EPSP amplitudes ($p = 0.05$; Figure 9D). As such, we conclude that the *Eph^{x652}* mutation has only a minor effect on baseline synaptic transmission. We also assessed whether the *Eph^{x652}* mutation disrupts synaptic growth. We find that bouton numbers in the *GluRIIA*; *Eph^{x652}* double mutant are not significantly different from the *GluRIIA* mutant alone ($p > 0.2$, data not shown; $N > 10$). Thus, the Eph receptor does not appear to have a role in synapse specification in the peripheral neuromuscular system, or in the developmental growth of the NMJ.

To assess the function of the Eph receptor during synaptic homeostasis we recorded from the NMJs of homozygous *GluRIIA^{SP16}*; *Eph^{x652}* mutant animals. In these double mutants, mepsp amplitudes are significantly decreased, as observed in *GluRIIA^{SP16}* mutants alone (Figure 9F). However, the average homeostatic increase in presynaptic release observed in the *GluRIIA* mutants is significantly suppressed in the *GluRIIA*; *Eph^{x652}* double mutant (Figure 9G;

$p=0.05$). In this analysis we normalize each experimental genotype to the appropriate genetic control. Control genotypes (wt, *Eph*^{X652}, *exn/+*; *Eph/+*) are normalized to themselves (100%) whereas experimental genotypes are normalized to the appropriate control (*GluRIIA* is normalized to wt, while *GluRIIA*; *Eph* is normalized to *Eph* and the *GluRIIA*; *exn/+*; *Eph/+* genotype is normalized to *exn/+*; *Eph/+*). This method of normalization takes into account the slight defect in baseline synaptic transmission. Thus, we can conclude that homeostatic compensation is significantly disrupted in the *Eph* mutant over and above the slight disruption of baseline synaptic transmission (see also supplemental Table 1). However, homeostatic compensation is not completely blocked.

Next, we sought additional evidence to support a role for the *Eph* signaling in synaptic homeostasis. We took advantage of a previously published dominant negative *Eph* receptor (UAS-*EphDN*) that includes the extracellular domain but lacks the cytoplasmic signaling domains (Dearborn et al., 2002). Neuronal expression of UAS-*EphDN* significantly disrupts synaptic homeostasis (Figure 9H, I). By contrast, muscle-specific expression of UAS-*EphDN* does not significantly impair synaptic homeostasis. It should be noted, however that muscle specific expression of UAS-*EphDN* does impair baseline synaptic transmission (see supplemental Table 1). These data are consistent with the conclusion that *Eph* could signal upstream of presynaptic *ephexin* during synaptic homeostasis. Finally, we took advantage of our previous observation that synaptic homeostasis is sensitive to partial losses of gene function (Figure 8). Here we show that a double-heterozygous combination of the *exn/+* and *Eph/+* mutants significantly disrupts synaptic homeostasis, while neither heterozygous mutation alone disrupts synaptic homeostasis (Figure 9F, G). These data provide further support for the conclusion that *Eph* receptors could signal with *Ephexin* during the process of homeostatic compensation.

DISCUSSION

Considerable evidence has accumulated to support the existence of a homeostatic signaling system at the *Drosophila* NMJ that originates in muscle and feeds back to modulate neurotransmitter release from the presynaptic nerve terminal (Davis, 2006). Here, we significantly advance our molecular understanding of this fundamental form of synaptic plasticity. We provide evidence that the *Drosophila* *Eph* receptor, *Exn* and *Cdc42* are core components of a homeostatic signaling system at the *Drosophila* NMJ. We also provide evidence that this signaling system ultimately converges upon *Ca*_v2.1 calcium channels, representing a plausible mechanism for the modulation of presynaptic vesicle release.

Recent evidence demonstrates that the *Drosophila* *Eph* receptor is neuronally expressed (Boyle et al., 2006). Additional evidence indicates that the *Drosophila* *Ephrin* homologue is expressed in *Drosophila* muscle (Tsuda et al., 2008). Based upon our data and these additional studies, we present a model for homeostatic signaling at the *Drosophila* NMJ. We hypothesize that the *Eph* receptor functions as a presynaptic receptor for a homeostatic, retrograde signal derived from muscle. The activated *Eph* receptor signals via *Exn* and *Cdc42* to eventually achieve increased calcium influx via the *Ca*_v2.1 calcium channel and, thereby, alter presynaptic release.

It is somewhat surprising that mutations in the *Eph* receptor only partially disrupt synaptic homeostasis. There are several possible explanations. One possibility is that the *Eph*^{X652} deletion mutation we have used is not a molecular null. This possibility has recently been suggested by an RT-PCR analysis (Tsuda et al., 2008). Another possibility is that the *Eph* receptor is an important component of homeostatic signaling, but is not the only presynaptic receptor that participates in homeostatic retrograde signaling at the NMJ. This would be consistent with previously proposed models of synaptic homeostasis based upon the use of multiple feedback signaling systems functioning in parallel (Davis, 2006). In either case, our

data implicate the Eph receptor and downstream signaling as important components of the homeostatic signaling systems that control presynaptic neurotransmitter release.

If the *Drosophila* Eph receptor functions to detect a retrograde signal at the NMJ during synaptic homeostasis, then there exist two prominent possibilities for the identity of the homeostatic retrograde signal. One candidate is *Drosophila* Ephrin, recently shown to be expressed in *Drosophila* muscle (Tsuda et al., 2008). Another candidate suggested by recent work in *C. elegans* and *Drosophila* is Vap33. The Vap33 protein can be cleaved and secreted and can function as an Eph receptor ligand during *C. elegans* fertilization. Vap33 has been placed at the *Drosophila* NMJ both pre- and postsynaptically, making it a candidate for a muscle-derived retrograde signal (Pennetta et al., 2002). However, unlike mutations in *Drosophila* *Eph* and *exn*, *Vap33* mutations cause a severe perturbation of NMJ growth and morphology (Pennetta et al., 2002). As such, Vap33 may be involved in a separate signaling system, or may have diverse functions in addition to those involved in synaptic homeostasis and in addition to those mediated by the putative synaptic Eph receptor.

Molecular mechanisms underlying the rapid induction versus sustained expression of synaptic homeostasis

It is interesting to note that *exn* mutations have a greater effect on synaptic homeostasis when placed in the *GluRIIA* mutant background compared to application of sub-blocking concentrations of PhTx, which cause a similar decrease in postsynaptic mepsp amplitude. PhTx-dependent signaling induces a homeostatic increase in presynaptic release in a time frame of seconds to minutes (Frank et al., 2006). By contrast, the *GluRIIA* mutant represents a persistent stress that requires a continuous increase in presynaptic release for several days. One possibility is that the *GluRIIA* mutant alters NMJ development in some fundamental manner to permanently compensate for decreased postsynaptic excitability, and this is the process most directly affected by the *exn* mutation. Several lines of evidence suggest that this is not the case. *GluRIIA* mutants have normal bouton numbers, normal muscle size (Petersen et al., 1997) and normal active zone number, estimated by quantification of the T-bar associated protein Bruchpilot as well as ultrastructurally (GWD and Richard Fetter, unpublished data). Thus, there is no evidence for a compensatory change in the anatomy or architecture of the *GluRIIA* NMJ. The homeostatic increase in vesicle release probability also seems very similar in PhTx treated animals and *GluRIIA* mutants. Both conditions have increased quantal content (estimated by average EPSP/average mepsp and by the method of failures; Petersen et al., 1997; Frank et al., 2006). In addition, both conditions show pronounced synaptic depression during prolonged stimulation (Frank et al., 2006), arguing that PhTx and the *GluRIIA* mutant both induce a similar change in presynaptic release probability and there has been no further developmental compensation in the *GluRIIA* mutant background. However, by analogy with the molecularly distinct, sequential phases of long-term potentiation (LTP) it remains possible that there will be molecular distinctions between the rapid induction and sustained expression of homeostatic signaling at the *Drosophila* NMJ. Eph-Exn signaling may represent one such molecular distinction.

Thus, we consider two scenarios. One possibility is that the Exn is not the only GEF or signaling molecule downstream of the Eph receptor involved in synaptic homeostasis. Consistent with this possibility, we find that both *Rho1/+* and *Rac1/+* heterozygous mutations disrupt synaptic homeostasis in the *GluRIIA* mutant background but do not interact genetically with *exn* during synaptic homeostasis. Thus, there may be sufficient signaling capacity in this GTPase system to induce a rapid homeostatic response following PhTx application. However, a perturbation that persists for several days such as the *GluRIIA* mutation, may place a burden on the homeostatic signaling system and reveal a requirement for Eph-Exn signaling. This would be possible if homeostatic signaling did not cause a persistent change to the presynaptic terminal,

but had to be continually updated during a persistent stress such as the *GlurIIIA* mutation. Alternatively, it is possible that Eph-Exn signaling is not the retrograde signal that is required for the rapid induction of synaptic homeostasis. Rather, this represents a retrograde signaling system that is primarily invoked to consolidate or sustain homeostatic signaling over time. In this scenario, Eph-Exn signaling could be considered a form of feedback signaling that ensures an appropriate level of homeostatic signaling is sustained over time. By analogy with homeostatic signaling systems in other tissues and other organisms, it has been proposed that multiple types of trans-synaptic feedback regulation will be necessary to execute and sustain an appropriate homeostatic signaling response (Davis, 2006).

Rho-GTPase signaling and the modulation of presynaptic transmitter release

Our data define a role for Rho-type GTPases in the modulation of vesicle release at a fast glutamatergic synapse. Rho-GTPases play a prominent role in many biological processes including regulation of the actin cytoskeleton and protein trafficking (Ridley, 2006). However, relatively little is known about the function of Rho-type GTPases during the regulation of calcium channel function. Rac1 and Cdc42 have been implicated in modulation of calcium channel function downstream of the Bradykinin receptor (Wilk-Blaszczak et al., 1997), but little else is known. By contrast, there is considerable evidence that the RGK family of small GTP binding proteins including Rad, Gem, Rem and Rem2 function to control calcium channel trafficking (Correll et al., 2008; Jarvis and Zamponi, 2007). The RGK family members all contain a Ras-like GTPase core and, like Rho-GTPases, are members of the Ras-GTPase superfamily. The RGK family members have been implicated in regulation of L-type calcium channel surface expression through the modulation of channel trafficking from the ER (Correll et al., 2008; Jarvis and Zamponi, 2007). It is interesting to speculate that Rho-GTPase signaling downstream of the Eph receptor could be involved in similar trafficking of Ca_v2.1 type calcium channels.

There is also evidence for Rho-GTPase signaling in the regulation of vesicle release. For example, Rho-GTPases including Cdc42 have also been implicated in the mechanisms of secretory granule fusion (Malacombe et al., 2006). In these studies, it is often hypothesized that the Rho-GTPases act primarily on the cytoskeleton and indirectly affect vesicle release. However, recent studies in *C. elegans* provide a different view. In these studies, Rho-GTPase signaling appears to modulate synaptic transmission via two independent pathways. In one pathway, RHO-1 inhibits DGK-1 (diacylglycerol kinase), leading to increased formation of DAG. Increased DAG is believed to recruit UNC-13 to the synapse and, thereby, potentiate synaptic transmission (Hiley et al., 2006; McMullan et al., 2006). There is also evidence for RHO-1-dependent modulation of presynaptic release that is independent of DAG and UNC-13, though this signaling pathway remains to be defined (Hiley et al., 2006; McMullan et al., 2006).

Here we provide evidence that Exn and Cdc42 are involved in the homeostatic modulation of presynaptic release. There is an important distinction to be made with respect to prior studies cited above. We have not eliminated the function of any Rho-GTPase in our assays. Rather, we demonstrate that the mechanisms of synaptic homeostasis are highly sensitive to changes in the level of Rho-GTPase signaling. Consistent with this fact, we do not find a dramatic alteration in the development of the NMJ and there is no change in active zone number in the *exn* mutant. By contrast, loss of other putative actin-regulatory proteins present at the *Drosophila* NMJ such as NSF (Stewart et al., 2002; Stewart et al., 2005), Nervous-wreck (Coyle et al., 2004), Dap160/Intersectin (Marie et al., 2004) and Profilin (unpublished data) cause a dramatic change in synapse morphology including an increase in the formation of small, highly branched synaptic boutons termed satellite boutons. Since we do not find any substantial or consistent correlation between a change in NMJ anatomy and impaired synaptic

homeostasis, we do not think that the *exn*, *Cdc42/+* (or *Rho1/+* or *Rac1/+*) mutations used in our study drastically affect the presynaptic actin cytoskeleton during NMJ development (see Rodal et al., 2008). Thus, we do not favor the hypothesis that Eph-Exn signaling acts primarily on the cytoskeleton with secondary effects on synaptic homeostasis.

At present, our data do not allow us to conclude whether changes in Ca_v2.1 channel conductance, abundance or organization are the primary target of Exn signaling and synaptic homeostasis. Although not definitive, we do present some evidence to suggest that modulation of Ca_v2.1 channel abundance may be involved in synaptic homeostasis. Specifically, we show that presynaptic expression of *UAS-Cac-GFP* partially restores synaptic homeostasis in an *exn* mutant. The simplest interpretation, though certainly not the only interpretation, is that we have restored channel abundance and that loss of channel abundance is the cause of the *exn*-mediated defect in synaptic homeostasis. This would be consistent with a mild defect in synaptic transmission without a change in release cooperativity in the *exn* mutant animals. According to this idea, *exn* mutations may limit the available Ca_v2.1 protein and prevent any subsequent homeostatic increase in channel abundance. Exn-Cdc42 signaling could alter the trafficking of Ca_v2.1 channels as suggested by the work on RGK GTPases (see above). Alternatively, Exn-Cdc42 signaling could regulate the availability of presynaptic sites for channel incorporation at the active zone, a possibility suggested by the “slot” model for calcium channel scaffolding at vertebrate central synapses (Cao et al., 2004).

MATERIALS AND METHODS

Electrophysiology

Recordings were taken from muscle 6 in abdominal segment 2 or 3 of third instar larvae as previously described (Davis et al., 1998). Recordings were made in HL3 saline with the following components (and concentrations): NaCl (70 mM), KCl (5 mM), MgCl₂ (10 mM), NaHCO₃ (10 mM), Sucrose (115 mM = 3.9%), Trehalose (4.2 mM = 0.16%), HEPES (5.0 mM = 0.12%), and CaCl₂ (0.5 mM unless specified). Quantal content was estimated for each recording by calculating the average EPSP/average mepsp. Quantal contents were calculated for each recording and then averaged across all NMJs for a given genotype. To assess calcium cooperativity, quantal contents were corrected for nonlinear summation according to established methods (Martin, 1955; Davis et al., 1996). For acute pharmacological homeostatic challenge, Philanthotoxin-433 (Sigma Aldrich) was used, as previously described (Frank et al., 2006). For divalent cation blockade of Ca²⁺ channels, CdCl₂ or NiCl₂ were used at specified concentrations.

Synapse Morphology and Anatomical Analyses by Immunocytochemistry

Third instar larval preparations were fixed for 2 min in Bouin's fixative (Sigma Aldrich) and incubated overnight at 4° C with primary antibodies. Secondary antibodies were applied for 2 hours at room temperature. The following antibodies were used: mouse anti-Brp 1:100 (Wagh et al., 2006); mouse anti-Synapsin 1:50 (Developmental Studies Hybridoma Bank, Iowa); mouse anti-GFP 1:500 (Molecular Probes) and rabbit anti-GluRIIC 1:1000 (Marrus et al., 2004). Alexa conjugated secondary antibodies and Cy3 conjugated goat anti-HRP were used at 1:200–1:800 (Jackson Immunoresearch Laboratories, Molecular Probes). Larval preparations were mounted in Vectashield (Vector) and imaged at room temperature using an Axiovert 200 (Zeiss) inverted microscope, a 100x Plan Apochromat objective (aperture 1.4), and a cooled CCD camera (Coolsnap HQ, Roper). Intelligent Imaging Innovations (3I) software was used to capture, process, and analyze images.

Generation of *UAS-YFP-exn*

The *UAS-YFP-exn* construct was generated by amplifying the full length *exn* open reading frame using the *GH03693* cDNA template (Drosophila Genomics Resource Center, Indiana) and then direct cloning into the UAS-N-terminal Venus (EYFP) vector (T. Murphy, Drosophila Genomics Resource Center, Indiana) using the Gateway™ cloning system (Invitrogen). The following primers were used to amplify the *exn* open reading frame: 5'-CACCATGTCGGCGTTGAATCGCAGCAA and 5'-TCACTTGCTCTTTTGC GACTCCAGGTAGG. The construct was confirmed by sequencing. Transgenic flies were generated by standard injection methods. The yeast two-hybrid binding assay between Exn and Eph was conducted using standard techniques and reagents (Golemis et al., 2008; Terman et al., 2002). The protein domains tested for interaction are as indicated (Figure 9). PCR amplification of DNA encoding the protein domains was done using the *exn* cDNA *GH03693* as a template (Drosophila Genomics Resource Center, Indiana).

Genetics and Drosophila Husbandry

Ephexin was identified as a gene involved in synaptic homeostasis using an assay to screen for genes that when mutated or knocked down cause impaired motility or development of the *GluRIIA* mutant animals. Genes were screened blind to identity and rescreened before selection and identification. Subsequently, genes identified in the first round of screening were examined for electrophysiological defects in synaptic homeostasis in the *GluRIIA* mutant background.

Drosophila melanogaster stocks with the following mutations, *UAS* transgenes, or *GAL4* drivers were used in the course of this study. **Chromosome X:** *w¹¹¹⁸* (Hazelrigg et al., 1984), *cac^S* (Smith et al., 1998), *Cdc42²* (Fehon et al., 1997; Genova et al., 2000), *Cdc42³* (Fehon et al., 1997; Genova et al., 2000), *Cdc42⁴* (Fehon et al., 1997; Genova et al., 2000), *C155 (elaV)-GAL4* (Lin and Goodman, 1994), *cac^{HC129}* (Kawasaki et al., 2002). **Chromosome II:** *GluRIIAS^{SP16}* (Petersen et al., 1997), *Rho1⁷²⁰* (Strutt et al., 1997), *Rho1^{k02107b}* (Magie et al., 1999), *UAS-YFP-exn^{FL10a}* (this study), *UAS-YFP-exn^{FL4}* (this study). **Chromosome III:** *exn^{EY01953}* (BDGP gene disruption project) (Bellen et al., 2004), *exn^{EY-Δ23}* (this study), *exn^{EY-Δ50}* (this study), *exn^{EY-5}* (this study), *Rac1^{J11}* (Hakeda-Suzuki et al., 2002; Ng et al., 2002), *BG57-GAL4* (Budnik et al., 1996), *UAS-YFP-exn^{FL2}* (this study), *UAS-EphDN* (Dearborn et al., 2002). **Chromosome IV:** *Eph^{X652}* (Boyle et al., 2006). Precise and imprecise excisions were generated using standard protocols and Δ2–3 as a transposase source. Animals were kept in chambers with controlled temperature and humidity (Forma Scientific). Experimental and control animals were reared in parallel and treated identically in all experiments. Homeostatic challenge was provided by the *GluRIIAS^{SP16}* null mutation (Petersen et al., 1997). The *w¹¹¹⁸* strain (Hazelrigg et al., 1984) was used as a wild-type control genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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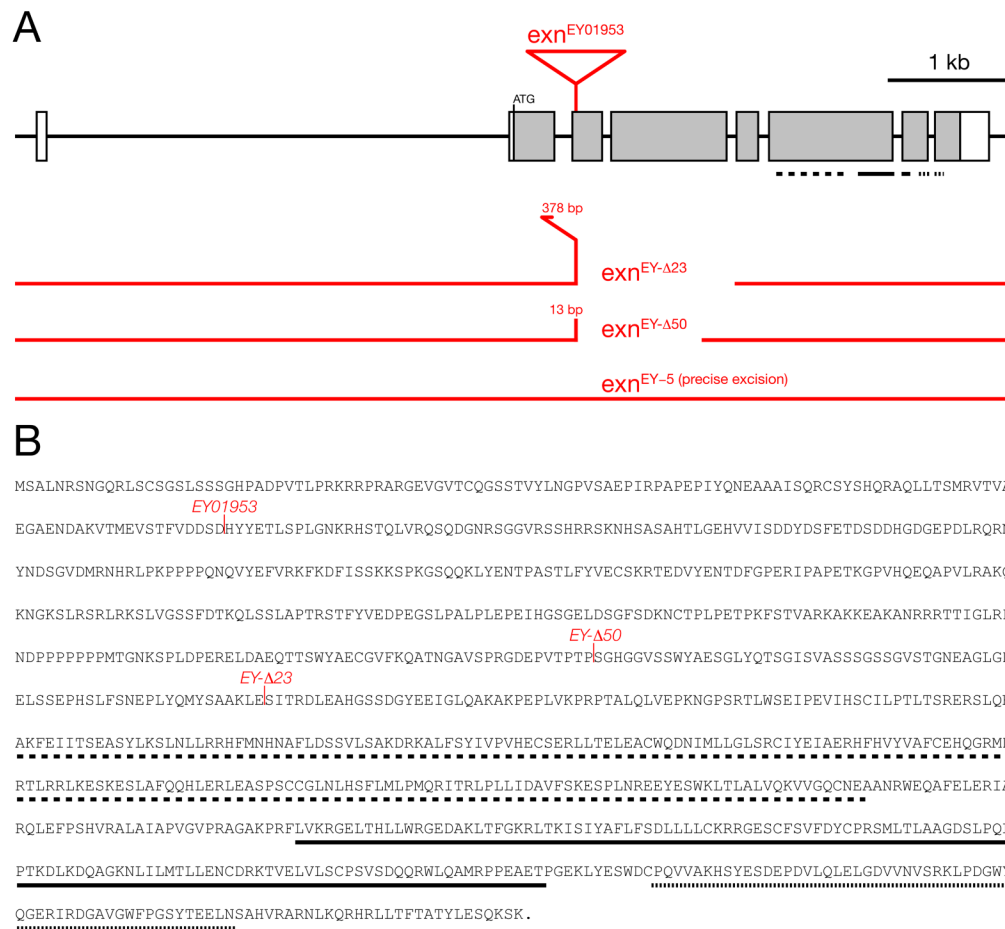


Figure 1. Organization and mutation of the *Drosophila exn* locus

A) Genomic overview of the *exn* gene. The insertion site of the P-element *exn*^{EY01953} and the extent of the deletions in the excision alleles *exn*^{EY-Δ23} and *exn*^{EY-Δ50} are indicated. The allele *exn*^{EY-5} is a precise excision of the *exn*^{EY01953} P-element insertion. **B)** Primary amino acid sequence of the Exn protein. *exn* encodes a 1051 amino acid long protein that contains a RhoGEF domain (dashed line), a PH domain (solid line) and an SH3 domain (dotted line). Domain location is also indicated in (A). The insertion of *exn*^{EY01953} and the distal break points of the *exn*^{EY-Δ23} and *exn*^{EY-Δ50} deletions are indicated.

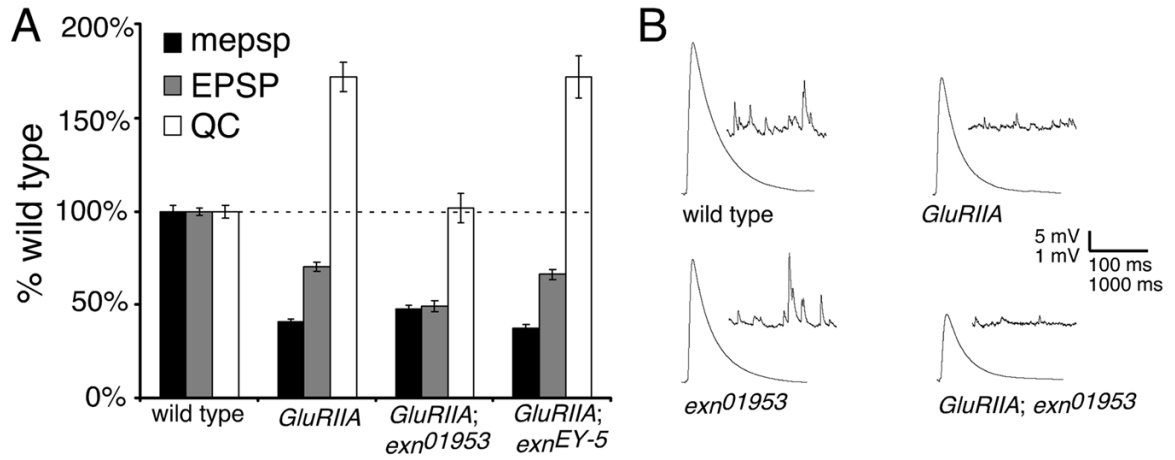


Figure 2. Impaired synaptic homeostasis at the *exn* mutant NMJ

A) Average values for mepsp (black), EPSP (gray), and quantal content (white) relative to wild-type control. The *GluRIIA^{SP16}* mutation causes a decrease in quantal size (mepsp). Decreased mepsp amplitude is partially offset by a homeostatic increase in presynaptic release (QC) ($p < 0.001$; Student's T-test). The *GluRIIA^{SP16}; exn^{EY01953}* double mutant NMJs show a decrease in mepsp amplitude without a corresponding increase in QC ($p > 0.2$ compared to wild type). A genotype composed of the precise excision allele (*EY-5*) and *GluRIIA^{SP16}* shows a robust homeostatic increase in QC ($p < 0.001$) similar to *GluRIIA^{SP16}* alone ($p > 0.9$ compared to *GluRIIA^{SP16}* alone). We also note a significant increase in mepsp amplitude comparing *GluRIIA^{SP16}; exn^{EY01953}* to the revertant *GluRIIA^{SP16}; exn^{EY-5}* ($p < 0.05$). **B)** Representative electrophysiological traces as indicated. Statistically significant differences are calculated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM). Data relevant to this figure are also presented in supplemental table 1.

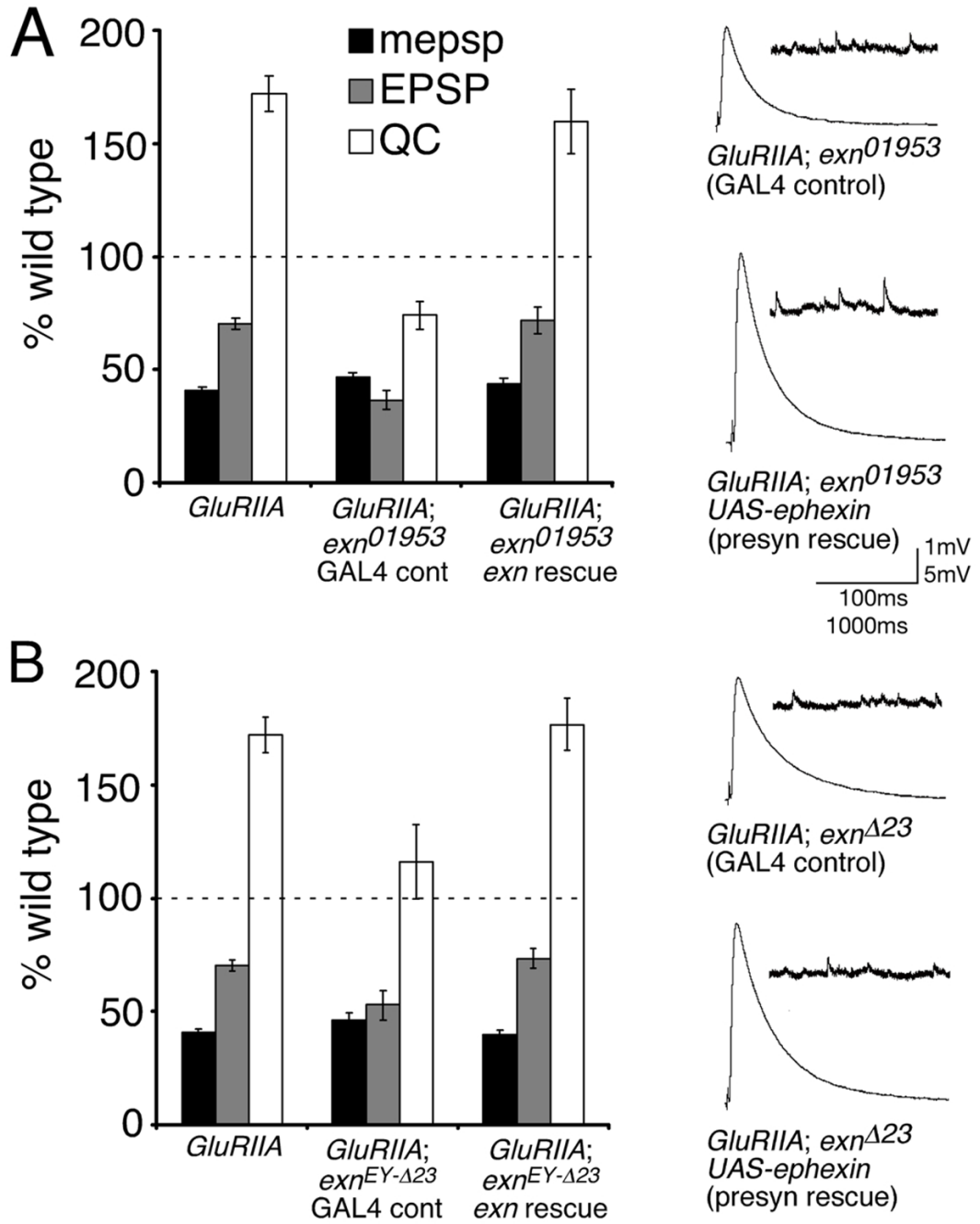


Figure 3. Exn is sufficient presynaptically for synaptic homeostasis

A) Average values for mepsp (black), EPSP (gray), and quantal content (QC; white) relative to wild-type control. Values are presented for *GluRIIA^{SP16}* alone, *GluRIIA^{SP16}; exn^{EY01953}* double mutants bearing a *UAS-YFP-exn* construct driven by the presynaptic *elav-GAL4* driver (*exn* rescue), or for sibling-matched controls with no driver (GAL4 control). Expression of *UAS-YFP-exn* significantly restores the homeostatic increase in release ($p < 0.001$ compared to sibling matched GAL4 control). Representative traces are shown at right. **B)** Values presented as in (A). Experiments are performed as in (A) except that the *exn^{EY-Δ23}* mutation is used. Representative traces are shown at right. Data are presented as average values (\pm SEM). Data relevant to this figure are presented in supplemental table 1.

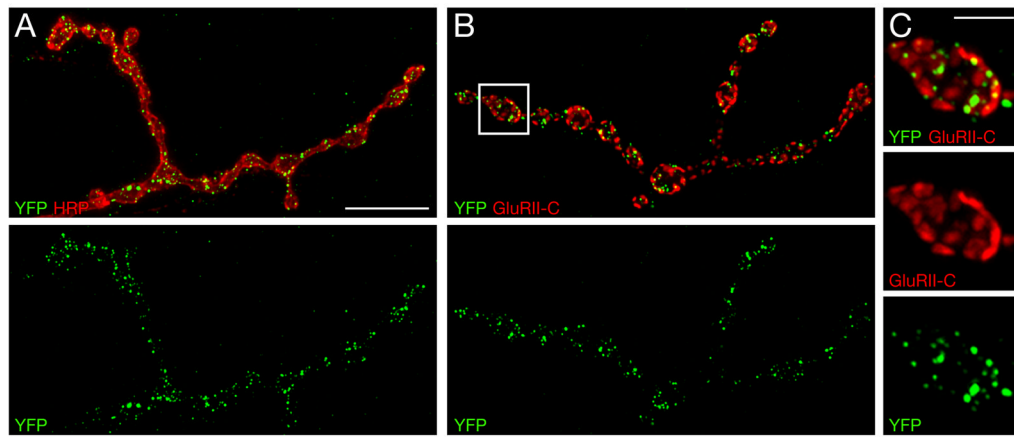


Figure 4. YFP-Exn distribution within the presynaptic nerve terminal

A–C) Overexpression of *YFP-exn* in motoneurons. A) NMJ co-stained for YFP-Exn (green) and presynaptic membranes (HRP, red). YFP-Exn is present in discrete puncta that are distributed throughout the presynaptic nerve terminal. B and C) The NMJ co-stained for YFP (green) and postsynaptic glutamate receptors (GluRII-C, red). C) Higher magnification of the boxed area in (B) shows accumulation of YFP-Exn opposite postsynaptic GluRII-C clusters. Scale bar in (A)–(B) 10 μ m. Scale bar in (C) 2.5 μ m.

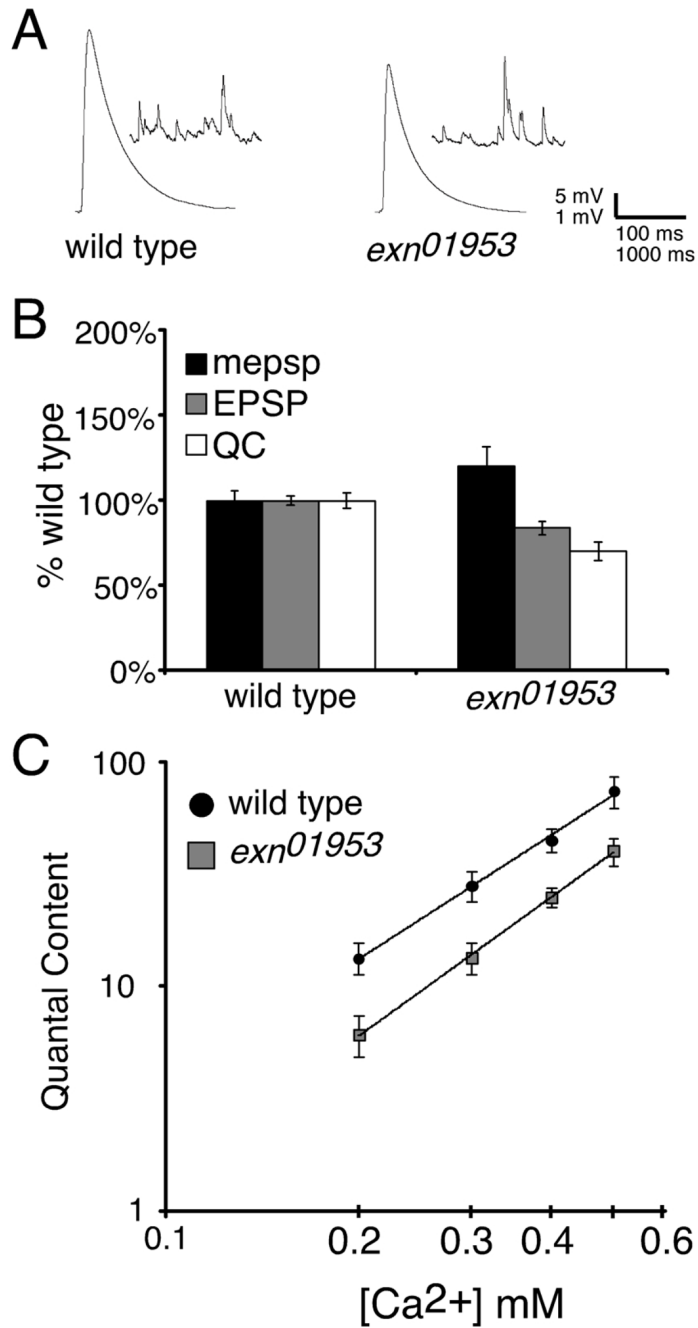


Figure 5. Impaired presynaptic release without a change in release cooperativity in *exn^{EY01953}* mutants

A) Representative electrophysiological traces of wild-type and *exn^{EY01953}* NMJs. **B)** Average values for mepsp (black), EPSP (gray), and quantal content (white) relative to wild-type control. *exn^{EY01953}* mutant NMJs display a small, yet significant deficit in neurotransmission ($p < 0.05$). **C)** Quantal content was quantified for wild type (black) and *exn^{EY01953}* (gray) over the range of extracellular calcium concentrations indicated. Statistically significant differences are indicated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM).

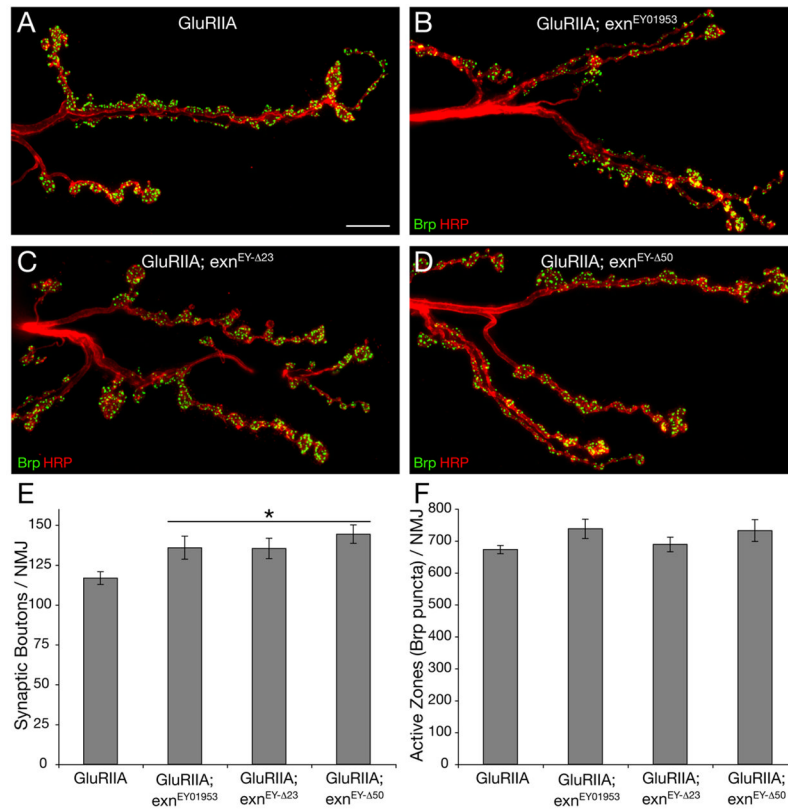


Figure 6. Normal active zone number at *exn* mutant NMJ

A–D) Partial view of muscle 6/7 NMJs in segment A2 co-stained for the presynaptic active zone protein Brp (green) and the presynaptic membrane marker HRP (red) for the genotypes indicated. Gross NMJ morphology is normal in each mutant. **E)** Quantification of the number of synaptic boutons at muscles 6/7 in segment A2. All *exn* mutations lead to a slight but significant ($p < 0.05$) increase in bouton number compared to *GluRIIA* mutations alone ($n \geq 8$). **F)** Quantification of the number of presynaptic active zones marked by Brp staining of muscle 6/7 NMJs in segment A2 (see text). There is no significant difference in the total number of Brp-positive active zones in *exn* mutants compared to the *GluRIIA* control background ($n \geq 9$). Scale bar in (A)–(D) 10 μm . Statistically significant differences are indicated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM).

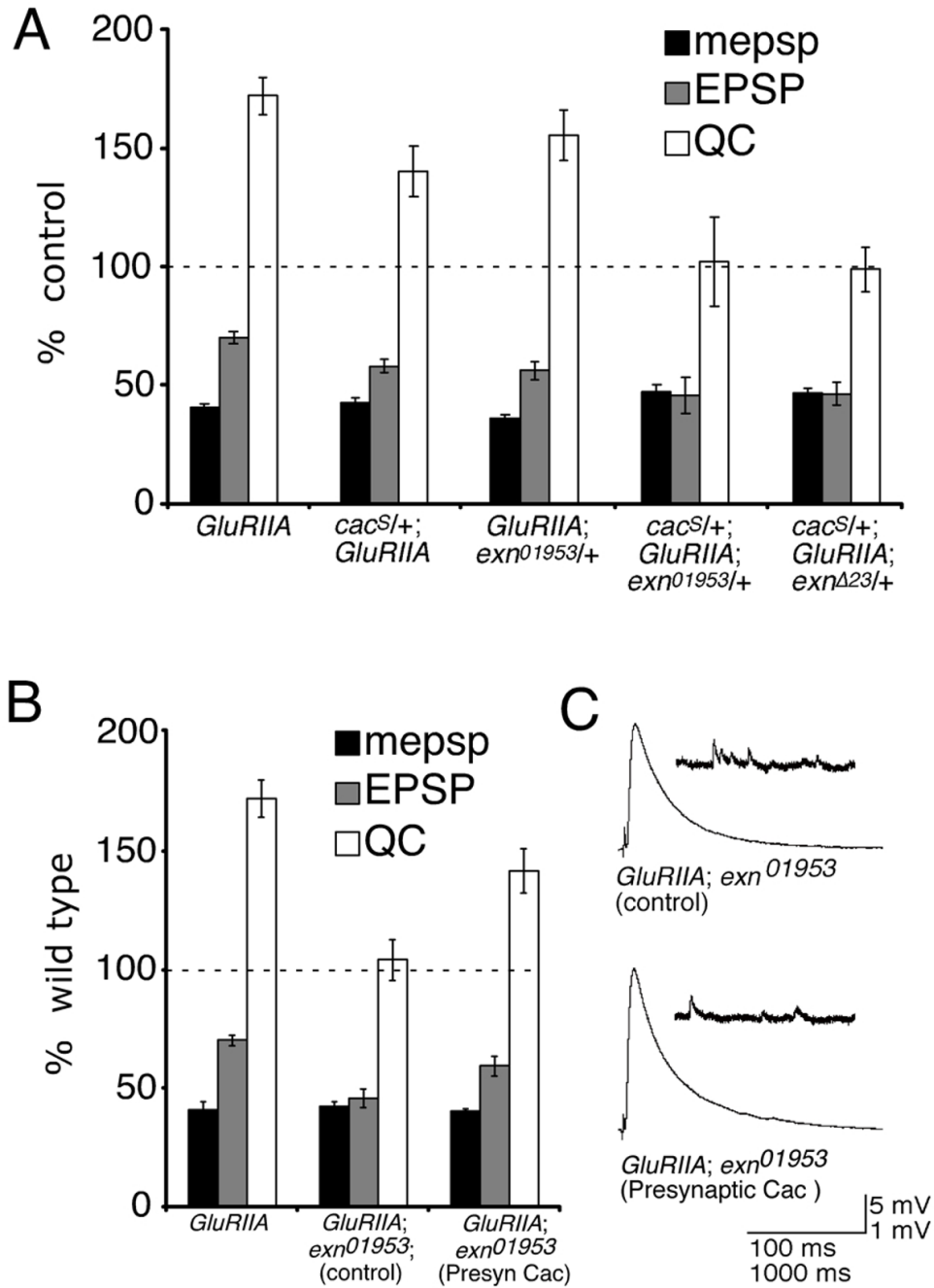


Figure 7. Genetic interaction between *exn* and presynaptic calcium channels encoded by *cac* ($Ca_v2.1$)

A) mepsp (black), EPSP (gray), and quantal content (QC; white) are plotted relative to genetic controls lacking the *GluRIIA* mutation. *cac^{S/+}; GluRIIA^{SP16}* and *exn^{EY01953/+}; GluRIIA^{SP16}* NMJs display robust homeostatic compensation that is slightly suppressed relative to control ($p < 0.05$ for *cac^{S/+}; GluRIIA^{SP16}*). By contrast, when a double heterozygous mutant combination is assayed in the *GluRIIA* mutant background (*cac^{S/+}; GluRIIA^{SP16}; exn^{EY01953/+}*) or (*cac^{S/+}; GluRIIA^{SP16}; exn^{EY-Δ23/+}*) the NMJs show no homeostatic increase in quantal content compared to control. **B, C**) Presynaptic expression of *GFP-cac* restores homeostatic compensation to the *GluRIIA; exn* double mutant. **B**) Average values as in **(A)**.

For the rescue experiment, values are presented for *GluRIIA*^{SP16}; *exn*^{EY01953} double mutants bearing a *UAS-GFP-cac* construct driven by the presynaptic *elav-GAL4* driver (presynaptic Cac), or for sibling-matched controls with no driver (control). In the absence of *elav-GAL4*, no rescue is observed (control). In the presence of *elav-GAL4*, a significant homeostatic increase in QC is observed ($p < 0.01$ compared to sibling matched controls). The level of homeostatic compensation does not reach *GluRIIA* alone ($p < 0.05$). C) Representative electrophysiological traces as indicated. Statistically significant differences are indicated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM). Data for (A) are presented in Supplemental Table 3. Data for (B) are presented in Supplemental Table 1.

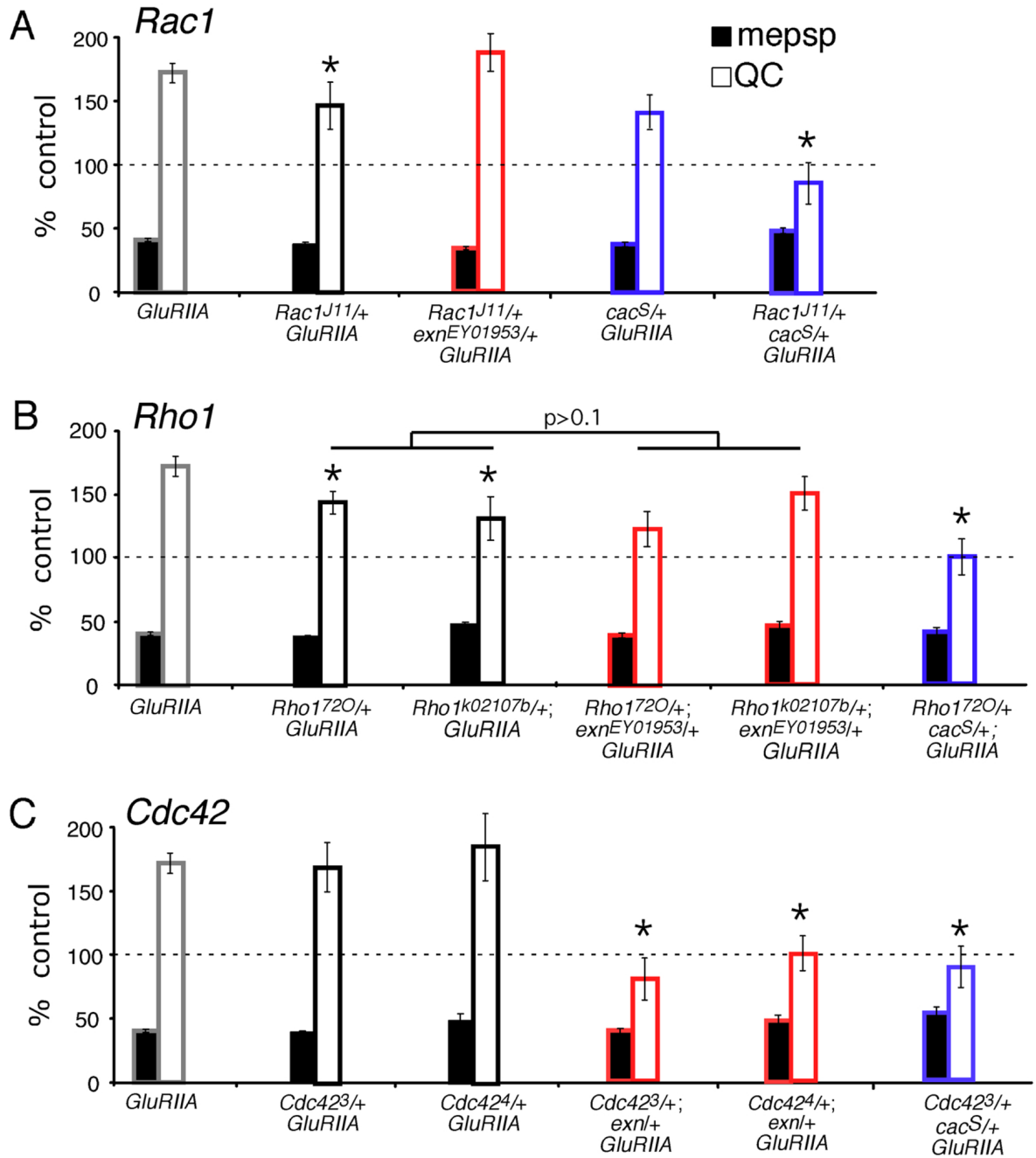


Figure 8. Rho-GTPases participate in the mechanism of synaptic homeostasis

Quantification for mepsp (black) and quantal content (QC; white) are plotted relative to control values in the absence of the *GluRIIA* mutation (100%). **A**) A heterozygous *Rac1^{J11/+}* mutant (black) has a slight, but significant ($p < 0.05$) decrease in synaptic homeostasis when compared to *GluRIIA* alone (black). This effect is not enhanced by a heterozygous *exn* mutation (*Rac1^{+/+}* with *exn^{+/+}* in the *GluRIIA* mutant background; red). The heterozygous *cac^S* mutation does not abolish homeostatic increase in QC in the *GluRIIA* mutant (blue). However, the double heterozygous *cac^{S/+} Rac1^{J11/+}* animals show a block of homeostatic compensation in the *GluRIIA* mutant background (blue, right). **B**) Heterozygous *Rho1^{+/+}* mutations (black) display a partial reduction in synaptic homeostasis (*; $p < 0.05$). This suppression of synaptic

homeostasis is not significantly enhanced by removing one copy of *exn* (red; $p > 0.1$). Synaptic homeostasis is completely blocked in double heterozygous *cac^{S/+}; Rho1/+* animals in the *GluRIIA* mutant background (blue; $p < 0.001$ compared to *GluRIIA* alone). **C**) Heterozygous *Cdc42/+* animals display a normal homeostatic increase in QC (black; $p > 0.9$ compared to *GluRIIA*). Homeostatic compensation is eliminated when the double heterozygous mutant combination of *Cdc42* and *exn* (red) are placed in the *GluRIIA* mutant background ($p < 0.001$ compared to *GluRIIA*) and when the double heterozygous combination of *Cdc42/+* with *cac^{S/+}* is placed in the *GluRIIA* mutant background (blue; $p < 0.001$ compared to *GluRIIA*). Statistically significant differences are indicated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM). Data relevant to this figure are presented in supplemental table 2.

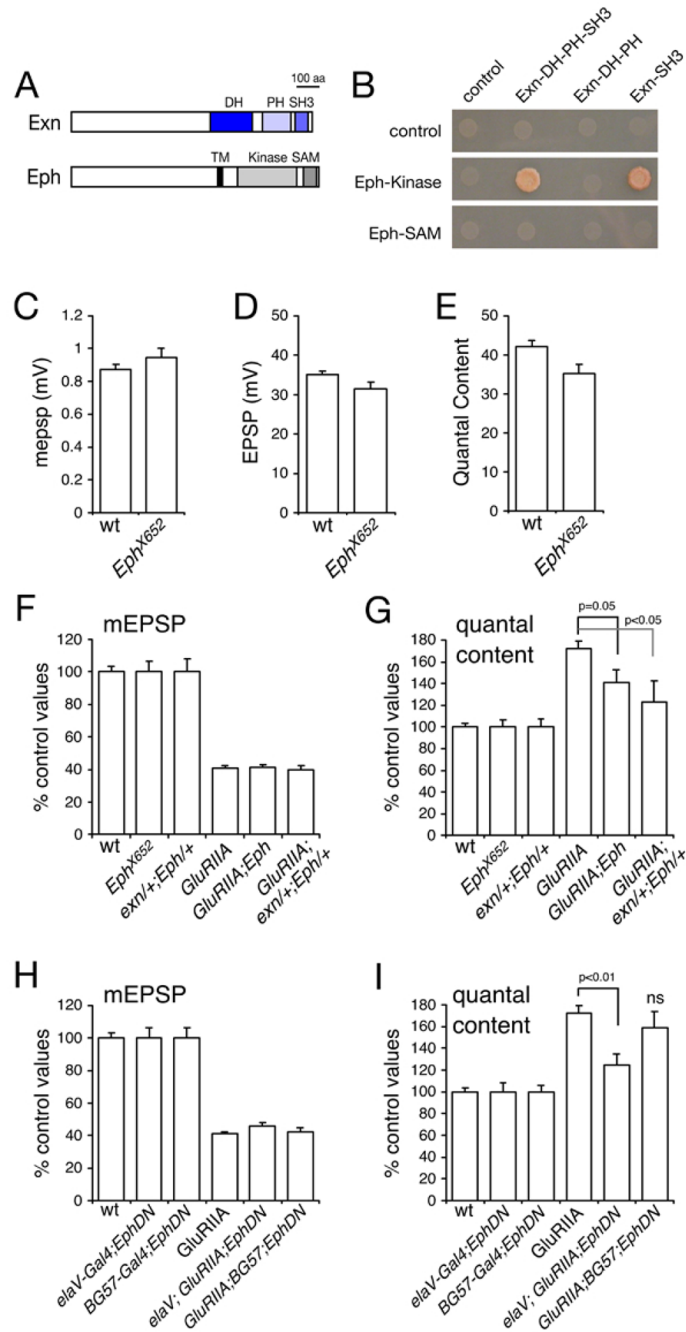


Figure 9. An Eph receptor mutation disrupts synaptic homeostasis

A) Diagrams of Drosophila Exn and Eph are shown at left. **B)** Data from a yeast two-hybrid system demonstrating an interaction between the Eph receptor kinase domain with constructs encoding either the DH-PH and SH3 domains of Ephexin or just the two single domains. **C–E)** Quantification of mEPSP amplitude (C), EPSP amplitude (D) and quantal content (E) comparing wild type and the *Eph^{X652}* mutant. A significant difference ($p < 0.05$) is found comparing EPSP amplitudes and comparing quantal contents. **F)** Quantification of mEPSP amplitudes for the indicated genotypes. Values are presented as normalized to values for each control genotype. Control genotypes (wt, *Eph^{X652}*, *exn/+; Eph/+*) are normalized to themselves (100%). Experimental genotypes are normalized to the appropriate control (*GluRIIA* is

normalized to wt. *GluRIIA; Eph* is normalized to *Eph. GluRIIA; exn/+; Eph/+* is normalized to *exn/+; Eph/+*). This normalization procedure takes into account any baseline transmission defect in the control genotype. All genotypes use *Eph^{X652}* though this notation is shortened in some columns for display purposes. **G**) Quantification of quantal content normalized to control genotypes as in (F). Statistically significant differences are indicated. **H**) Quantification of mEPSP amplitudes for the indicated genotypes. Data are normalized as in (F). For display purposes *elaV-GAL4* is shortened to *elaV* in column 4 and *BG57-GAL4* is shortened to *BG57* in column 5. **I**) Quantification of quantal content for the indicated genotypes (notation as in H). Data are normalized as in (F). Statistically significant differences are indicated according to an unpaired, two-tailed Student's T-test. Data are presented as average values (\pm SEM). Data relevant to this figure are presented in supplemental table 1.