Unc-51 Controls Active Zone Density and Protein Composition by Downregulating ERK Signaling

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Efficient synaptic transmission requires the apposition of neurotransmitter release sites opposite clusters of postsynaptic neurotransmitter receptors. Transmitter is released at active zones, which are composed of a large complex of proteins necessary for synaptic development and function. Many active zone proteins have been identified, but little is known of the mechanisms that ensure that each active zone receives the proper complement of proteins. Here we use a genetic analysis in Drosophila to demonstrate that the serine threonine kinase Unc-51 acts in the presynaptic motoneuron to regulate the localization of the active zone protein Bruchpilot opposite to glutamate receptors at each synapse. In the absence of Unc-51, many glutamate receptor clusters are unapposed to Bruchpilot, and ultrastructural analysis demonstrates that fewer active zones contain dense body T-bars. In addition to the presence of these aberrant synapses, there is also a decrease in the density of all synapses. This decrease in synaptic density and abnormal active zone composition is associated with impaired evoked transmitter release. Mechanistically, Unc-51 inhibits the activity of the MAP kinase ERK to promote synaptic development. In the unc-51 mutant, increased ERK activity leads to the decrease in synaptic density and the absence of Bruchpilot from many synapses. Hence, activated ERK negatively regulates synapse formation, resulting in either the absence of active zones or the formation of active zones without their proper complement of proteins. The Unc-51-dependent inhibition of ERK activity provides a potential mechanism for synapse-specific control of active zone protein composition and release probability.

Key words: autophagy; NMJ; synapse; Drosophila; ATG; active zones; glutamate receptors

Introduction

At the synapse, presynaptic release machinery is directly apposed to neurotransmitter receptors within the postsynaptic density. The active zone is the site of neurotransmitter release, and is composed of a large, macromolecular complex of proteins that are involved in the development and function of the synapse (Fejtova and Gundelfinger, 2006). While many active zone proteins have been identified, little is known of the signaling pathways that regulate the assembly of these proteins and ensure that each active zone receives the proper complement of proteins.

The Drosophila neuromuscular junction is a favorite model system for identifying mechanisms that shape the development and function of the synaptic terminal, but until recently had been little used for the study of active zones (Collins and DiAntonio, 2007). This changed with the identification and characterization of Bruchpilot (Brp), the fly ortholog of the vertebrate active zone protein CAST (Wagh et al., 2006). Bruchpilot is present at every active zone, and is required for the active zone localization of presynaptic calcium channels and T-bars, a dense body specialization thought to promote efficient transmitter release (Kittel et al., 2006). Each Drosophila NMJ comprises hundreds of active zones directly apposed to postsynaptic glutamate receptors, and these can be visualized with antibodies against Brp (Wagh et al., 2006) and the essential glutamate receptor subunit DGLuRIII (Marrus et al., 2004). While each of these active zones is formed by the same presynaptic motoneuron, they have a heterogeneous release probability that correlates with the abundance of Brp (Marrus and DiAntonio, 2004). This suggests that mechanisms exist to regulate the efficacy of individual release sites, potentially by controlling the protein constituents of the active zone. To identify such mechanisms, we performed a large-scale screen for genes required to ensure that each postsynaptic cluster of glutamate receptors is apposed to an active zone containing Bruchpilot.

Here we report that the serine threonine kinase Unc-51 functions in the presynaptic neuron to ensure that Bruchpilot is apposed to glutamate receptors at each synapse. In the absence of Unc-51, many glutamate receptor puncta are unapposed to Brp, and ultrastructural analysis demonstrates that fewer active zones contain T-bars. In addition to these aberrant synapses, many...
fewer synapses form in the mutant and there is a large deficit in evoked transmitter release. Mechanistically, Unc-51 promotes synaptic development by inhibiting the activation of the MAP kinase ERK. In the unc-51 mutant, excess ERK activity is responsible for the decreased synaptic density and the absence of Bruchpilot from each synapse. We propose a model in which activated ERK negatively regulates synapse formation, leading to the formation of active zones without Bruchpilot or, in more extreme cases, the complete absence of the synapse. This Unc-51-dependent downregulation of ERK activity is a potential mechanism for synapse-specific control of active zone protein composition and release probability.

Materials and Methods
Fly stocks. Flies were raised and maintained at 25°C on standard fly media. Wild-type flies were either Canton S (CS) outcrossed to w; elav-Gal4 (Yao and White, 1994), or G7-Gal4 (Zhang et al., 2001) based on the experiment. The following fly lines were obtained from the Bloomington/H9004 stock center: P-element collection on the third chromosome (Bellen et al., 2004), deficiency line: Df(3L)BSC10, P-element insertions: P[EPgy2]Atg1EY07351 CG17667EY07351, and P[EPgy2]Atg1EY09216 and P[EPgy2]Atg1EY07351 CG17667EY07351, respectively, toward downstream from the insertion point, deregulated parts of the first exon, including the ATG initiation codon. These lines were used for the experiments described in the Results section.

Western blots. Wandering third-instar larvae were dissected in ice-cold homogenization buffer (67 mM Tris-HCl, pH 8.0, 67 mM NaCl, 2 mM urea, 1 mM EDTA, and 1.3% SDS) and stored immediately on dry ice. The samples were run on 10% SDS-PAGE gels according to standard procedures. Mouse anti-diphospho ERK (Sigma, catalog #M8159) and mouse anti-phospho p38 (Cell Signaling Technology) were used at 1:1000. The blot was stripped using standard procedures and then probed for non-phosphorylated ERK-MAP kinase using rabbit anti-rolled antibody (Peverati et al., 1996) at 1:1000. Mouse anti-syntactin antibody (BC3) and mouse anti-β-tubulin (E7) antibody were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa. G7-Gal4 larvae were used at 1:100. HRP-conjugated goat anti-anti mouse secondary antibodies (Jackson ImmunoResearch) were used at 1:10,000.

Imaging and analysis. Imaging and analysis were performed as described previously (Viquez et al., 2006) with the following minor differences. The experimenter was blinded to genotypes during both imaging and analysis. Statistical analysis was performed using one-way ANOVA for comparison of samples within an experimental group. n for each condition is described either in the figures or in the results section. All histograms and measurements are shown as mean ± SEM. Bruchpilot (BRP) and DGLuRIIII puncta were manually counted at MN4b synapses on muscle 4. DGlulRIIIa puncta that were not opposite to a detectable BRP punctum were counted as unopposed DGlulRIIIa puncta. Area of NMJ was calculated by threshold function in MetaMorph for HRP channel. For the analysis of the axon transport phenotype, a box was drawn along a region of axon approximately between segments A3 and A4 and the same size box was used to measure the intensity of BRP in both mutants and wild type. HRP was used to threshold the area within the box. ImageJ was used to analyze the intensity of bands on the Western blots and the measure of protein expression was used to quantify the intensity of bands. Ratio of the intensity of diphospho (activated) ERK bands to that of syntaxin bands was calculated and used for calculating the statistical difference between the genotypes. One-way ANOVA was performed to determine significance of the difference between band intensities in an experimental group. For electron microscopy analysis, bouton circumference was measured using the free hand tool in MetaMorph and by measuring around the outer margin of the bouton. T-bars and active zones were readily recognizable due to their electron dense appearance. Both of them were manually counted. An active zone was recognized by electron dense region of a bouton that had the presynaptic and postsynaptic membranes tightly apposed to each other. A T-bar was defined as a membrane structure protruding from the electron-dense active zones and surrounded by vesicles.

Electrophysiology. Intracellular electrophysiological recordings were performed as previously described (Marrus et al., 2004). Briefly, wandering third-instar larvae were dissected in 0 mM CaCl2 Stewart saline (HL3) (Stewart et al., 1994). Both spontaneous miniature excitatory junction potentials (mEJPs) and evoked potentials (EPs) were recorded in either 0.4 or 0.42 mM HL3. Calcium concentration was identical for every experiment. At least 60 consecutive miniature events were measured per cell using MiniAnalysis (Synaptosoft) and averaged to determine mean mEJP. Events with a slow rise time were rejected as likely artifacts arising from neighboring electrically coupled muscle cells. To record evoked EPs, segmental nerves were cut and suctioned into a stimulating electrode, in which they received a brief depolarizing pulse. Quantal content was estimated by dividing the mean EJP by the mean mEJP (EJP/mEJP). Cells across all genotypes had similar mean input resistances and resting potentials. Quantal size was not significantly different among any of the genotypes compared with wild-type controls. Statistical analysis was performed using one-way ANOVA to compare samples in an experimental group.

Electron microscopy. The larval head and tail were pinned and a dorsal slit was made lengthwise, thus filling the larvae – in phosphate buffer. The larvae were then placed into fixative (2% paraformaldehyde/2.5% glutaraldehyde in cacodylate buffer, pH 7.3) o/n. The larvae were washed three times with cacodylate buffer. The larvae were dehydrated through 60 (1 ×, 7 min), 70, 80, 95 and 100% ETOH (2 ×, 10 min each step), transferred into propylene oxide (2 ×, 10 min), then into a 1:1 mixture of propylene oxide and Eponate, and left o/n, capped and at room temp. The larvae were then placed into fresh Eponate and into a mold, oriented and allowed to polymerize at 70°C. Thin sections were made and placed on Superfrost plus micro slides, heated on a hot plate.
Ahantarig et al., 2008). Atg1 participates in autophagy in transgenic P-element insertion lines (see Materials and Methods) within the intron of uncals and worms, clature, we will refer to the gene here by the name used in mammal CG17667, which many DGluRIII clusters are not apposed by a detectable BRP punctum. This P-element is inserted in Pegarn), a serine-threonine kinase that is the ortholog of Unc-51 from C. elegans and vertebrates (Scott et al., 2004; Tomoda et al., 1999; Scott et al., 2004; Tomoda et al., 2004), which is inserted within the first exon of the unc-51 gene (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) in mutants. Neuronal RNAi knockdown leads to unapposed DGluRIII puncta by the neuronal driver elav-Gal4 rescues all morphological phenotypes associated with unc-51 mutants. In the rescued mutant the synaptic defects are fully rescued: the density of DGluRIII puncta, the density of BRP puncta, and the apposition of Brp puncta to DGluRIII puncta are all returned to wild-type levels (Fig. 1C–E). In addition, the NMJ area is increased more than twofold compared with the mutant (Fig. 1B), while the axonal accumulations are decreased by about half (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Postsynaptic expression of the unc-51 transgene using G7-Gal4 driver does not rescue any of the defects (data not shown). Hence, unc-51 is required in the presynaptic motoneuron. In addition, selectively inhibiting Unc-51 function in the presynaptic cell via expression of a transgenic RNAi line pheno-
copies the unc-51 mutant. Neuronal RNAi knockdown leads to unapposed DGluRIII puncta, a reduction in synaptic density, a decrease in NMJ area, and accumulation of synaptic material in the axon including BRP and vesicle associated proteins like DVGLUT (supplemental Fig. 1, available at www.jneurosci.org as supplemental material, and data not shown), suggesting that the axon transport defect is not specific for a particular synaptic protein. Hence, the wild-type function of unc-51 is to act in the motoneuron to promote a high density of synapses, and to ensure that molecular constituents of the release site localize opposite to each postsynaptic density.

In some mutants with axon transport defects such as dynactin mutants, a smaller NMJ is observed and is due in part to retraction of the presynaptic terminal (Eaton et al., 2002). Such retraction could not explain the synaptic apposition defect of the unc-51 mutant, because unapposed DGluRIII puncta are present in a salt-and-pepper pattern throughout the NMJ, rather than only in the distal boutons as would be predicted for the progressive retraction of the synaptic terminal. However the small size of the NMJ in the mutant could be due to such retraction, so we assayed for synaptic retraction in the unc-51 mutant. The hallmark of synaptic retraction is the presence of "synaptic foot-
prints,” in which postsynaptic markers such as the PDZ-protein Dlg are unapposed to synaptic vesicle proteins or presynaptic membrane (Eaton et al., 2002). To test whether unc-51 mutants have a retraction phenotype, we stained wild type and unc-51 mutants for the synaptic vesicle protein DVGLUT and the presynaptic membrane marker HRP, as well as the postsynaptic marker Dlg. We did not observe synaptic footprints, even in severe cases in which the NMJ size was drastically reduced (Fig. 2). Hence, the small size of the NMJ in the unc-51 mutant is not due to a failure to maintain the presynaptic terminal at the NMJ, but instead suggests a defect in the development of the NMJ.

The density of active zones and T-bars is decreased in Unc-51 mutants

Our confocal analysis of synaptic structure makes several predictions about synaptic ultrastructure in the unc-51 mutant. First, immunoelectron microscopic studies of glutamate receptors reveal that glutamate receptors are clustered opposite every active zone (Petersen et al., 1997). Since the density of DGluRIII puncta is reduced in the mutant, we predict a decrease in active zone density. Second, Brp is necessary for the localization of the dense body T-bar to the active zone (Kittel et al., 2006). Since many DGluRIII puncta are unapposed to Brp, we predict that there will be fewer T-bars/active zone in the mutant. In addition, since the density of Brp puncta is decreased, we also expect a decrease in the density of T-bars. Electron microscopic analysis of wild-type and unc-51 mutant NMJs from type 1b boutons confirmed our predictions. In wild-type type 1b boutons are filled with vesicles and are surrounded by a subsynaptic reticulum, which is an extensive postsynaptic membrane-infolding (Fig. 3). Active zones are apparent as regions of increased electron density where the presynaptic and postsynaptic membranes are tightly apposed. The T-bar is a dense body specialization thought to promote release of transmitter that is observed as a T-like structure at a subset of active zones. Typically, synaptic vesicles are seen clustered near the

![Figure 1.Unc-51 is required for normal synaptic density and apposition. A, Sample confocal images of larval NMJs from muscle 4 stained with antibodies against BRP (red), DGluRIII (green), and HRP (blue). The genotypes of the larvae are as follows: WT (CS crossed to elav-GAL4), unc-51 deletion mutant, and unc-51 Rescue (elav-GAL4/UAS-unc-51; unc-51) mutants show magnified view of boutons. Note the many DGluRIII puncta (arrowheads) without a detectable BRP punctum in the mutant. B–E, Histograms of NMJ area (B), glutamate receptor density (C), BRP density (D), and ratio of BRP to DGluRIII (E) at WT, unc-51, and unc-51 Rescue NMJs, muscle 4. n = 10 for all the genotypes; ***p < 0.001 and **p < 0.01. Error bars represent SEM. Scale bar, 2 μm.](image-url)
T-bars. While the mutant the synaptic ultrastructure is broadly similar to wild type, with active zones, T-bars, and synaptic vesicles, we observed a number of quantitative phenotypes that are consistent with our light-level analysis. First, the density of active zones is decreased by \( \frac{1}{4} \) in the mutant (Fig. 3B), consistent with the \( \frac{1}{3} \) decrease in DGluRIII puncta measured at the light level. Second, unc-51 mutants showed a 33\% decrease in active zones containing a T-bar (Fig. 3C), which corresponds to the \( \frac{1}{3} \) of unapposed DGluRIII puncta seen with the confocal. Finally, the mutants also exhibit a >50\% decrease in the density of T-bars (Fig. 3D), which is in excellent agreement with the \( \frac{1}{3} \) reduction in the density of Brp puncta observed with light microscopy. Thus in the absence of unc-51, synaptic density is dramatically decreased and is accompanied by many abnormal active zones which lack T-bar specializations.

unc-51 mutants display defects in neurotransmitter release

The unc-51 mutant exhibits defects in both the number and structure of active zones, either of which could impair synaptic function. To investigate the strength of synaptic transmission in this mutant, we measured both evoked and spontaneous neurotransmitter release at the NMJ. We performed intracellular electrophysiological recordings from muscle 6, segment A2 or A3 from both wild type and unc-51 mutants (Fig. 4). Consistent with the morphological defects, there is an approximately threefold reduction in the amplitude of evoked excitatory junction potentials (EJPs) in two different allelic combinations of unc-51 mutants compared with wild type (Fig. 4C). A defect in EJP amplitude could be due to the release of fewer vesicles from the nerve, or to a decrease in postsynaptic sensitivity to transmitter. To distinguish between these possibilities, we recorded spontaneous miniature junctional potentials (mEJPs), which are a measure of the postsynaptic response to the fusion of a single synaptic vesicle. In contrast to evoked release, there is no significant difference in the amplitude of mEJPs in these genotypes (WT = 0.88 ± 0.08 mV, unc-51\(^3\) = 0.73 ± 0.06 mV, unc-51\(^3\)/16816 = 0.80 ± 0.03 mV, \( n = 10, p = 0.1 \)), suggesting a presynaptic defect in transmitter release. Quantal content is a
measure of the number of synaptic vesicles released by the pre-
synaptic terminal following an action potential. Calculating
quantal content by the direct method (EJP/mEJP) reveals a three-
to-fourfold reduction in the number of synaptic vesicles released
in the mutant (Fig. 4D). Consistent with a defect in presynaptic
release, we observe a decrease in the frequency of mEJPs in both
unc-51 mutants, although the difference is only statistically sig-
ificant in one genotype (WT = 2.4 ± 0.2, unc-51<sup>3</sup> = 2.0 ± 0.3
and unc-51<sup>3</sup>/16816 = 1.6 ± 0.2, p < 0.05 for WT; unc-51<sup>3</sup>/16816
and p = 0.3 for WT: unc-51<sup>3</sup>, n = 10). These findings demon-
strate that unc-51 is required for normal synaptic transmission,
potentially due to the reduced number of morphologically nor-
mal synapses in the mutant.

Transgenic expression of unc-51 rescues the morphological
phenotypes of the unc-51 mutant, so we expected that that it
would also rescue the physiological phenotypes. Surprisingly, we
were unable to rescue the quantal content defect (Fig. 4). Since
Unc-51 is a kinase, its overexpression may be deleterious and
hence obscure our efforts to observe rescue. To test this possibil-
ity, we overexpressed unc-51 presynaptically (elav-Gal4 crossed to
UAS-unc-51<sup>3</sup>-Rescue transgene). This demonstrates that the level of
unc-51 expression is important for synaptic transmission. Overexpres-

Figure 4. Evoked transmitter release is impaired in unc-51 mutants. A, Representative spontaneous mEJPs from the following genotypes: WT (CS or elav crossed to w<sup>+</sup>), unc-51<sup>3</sup> deletion mutants, hypomorphic allele unc-51<sup>3</sup>/16816 (unc-51<sup>3</sup>/P[EPgy2]Ay(VP16)59G1766 EY07351), elavGal4/unc-51<sup>3</sup>-rescue, unc-51<sup>3</sup>, and a wild-type larva overexpressing unc-51 presynaptically (elav-Gal4 crossed to UAS-unc-51<sup>3</sup>-Rescue transgene). B, Representative evoked EJPs from the same genotypes as in A. C-D, Histograms show the mean EJP amplitudes (C) and quantal content (D). Quantal
content was calculated by dividing the EJP amplitude by the mEJP amplitude for the same cell. ***p < 0.001. Error bars represent SEM and n = 9 for all genotypes.
Neuronal expression of \textit{unc-51} mutant larvae. Immunoblot analysis reveals an increase in the levels of dp-ERK in the \textit{unc-51} mutants that is rescued by the neuronal expression of \textit{unc-51} (dp-ERK: WT = 100\% ± 20\%, \textit{unc-51} \textit{d} = 184\% ± 13\%, \textit{unc-51} \textit{d} + rescue = 101\% ± 17\%, n = 3, p = 0.02). In these genotypes there is no change in the levels of total ERK (WT = 18.3 ± 2.6 AU, \textit{unc-51} \textit{d} = 15.3 ± 5.3 AU, rescue = 13 ± 4.1 AU, n = 3, p > 0.6) or syntaxin (WT = 41.3 ± 9.7 AU, \textit{unc-51} \textit{d} = 42.6 ± 6.6 AU, rescue = 45.3 ± 9.1 AU, n = 3, p > 0.9) (Fig. 5). Hence, \textit{unc-51} is required in vivo to inhibit ERK activity.

**Figure 5.** \textit{unc-51} inhibits ERK activation. Western blot probed using anti-diphospho (activated) ERK, anti-rolled and anti-syntaxin antibody. Activated ERK is increased in \textit{unc-51} mutants compared with wild type (n = 3, p < 0.05). Neuronal expression of \textit{unc-51} transgene in the mutant rescues the increase in activated ERK levels (n = 3, p < 0.05). Levels of ERK are not significantly different among the three genotypes (n = 3, p = 0.2). Genotypes used are as follows: WT (CS crossed to elav-GAL4), \textit{unc-51} \textit{d} deletion mutant, and \textit{unc-51} \textit{d} \textit{rescue} (elav-GAL4/\textit{UAS-unc-51; unc-51} \textit{d}).

**Mutations in ERK suppress the synaptic defects of \textit{unc-51} mutants**

If excess ERK activity contributes to the \textit{unc-51} synaptic phenotypes, then reducing ERK activity should suppress those defects. ERK null mutants are embryonic lethal (Biggs et al., 1994), so we generated a double mutant between \textit{unc-51} \textit{d} and the well characterized ERK (\textit{rolled}) hypomorph, \textit{rl} \textit{d}, that survives past the larval stage (Lim et al., 1999). We stained the NMJs of wild-type, \textit{unc-51} \textit{d} mutant, \textit{rl} \textit{d} mutant, and \textit{unc-51} \textit{d}, \textit{rl} \textit{d} double mutant larvae for the active zone component Brp and the glutamate receptor DGluriI. Quantitative analysis was performed at the identified NMJ formed by the Mnb4 motoneuron onto muscle 4, although results were qualitatively similar at all type 1 glutamatergic NMJs. There is no significant difference in the NMJ area, synapse density, or apposition between Brp and DGluriI in the ERK hypomorph \textit{rl} \textit{d} when compared with wild type. As shown above, \textit{unc-51} \textit{d} mutants display a decrease in synapse area, a decrease in synapse density and a high degree of misapposition between Brp and DGluriI. In the \textit{unc-51} \textit{d}, \textit{rl} \textit{d} double mutant there is dramatic suppression of each of these phenotypes (Fig. 6A–D). The double mutant shows a more than twofold increase in the NMJ area, and a complete suppression of the synaptic density and apposition phenotypes to wild-type levels. Hence, increased ERK activity is required for the \textit{unc-51} synaptic defects. There is an additional \textit{unc-51} phenotype, the accumulation of synaptic material in the axon that is indicative of an axon transport defect. Of note, this axon transport defect is not suppressed in the double mutant (Fig. 7A, B). Therefore, the axonal and synaptic defects of the \textit{unc-51} \textit{d} mutant are genetically separable, and so the transport defects cannot be responsible for the synaptic defects. Instead, these results suggest that \textit{unc-51} regulates at least two pathways. It is required to downregulate ERK activity to ensure proper synaptic development, and works via a second, unknown pathway to promote proper axonal transport.

Having demonstrated that \textit{rl} \textit{d} suppresses the synaptic morphology defects of the \textit{unc-51} \textit{d} mutant, we next tested whether it also suppresses the defects in synaptic function. Evoked and spontaneous synaptic events were recorded from muscle 6, segments A2/A3 in the same four genotypes described above. While synapse morphology is normal in the \textit{rl} \textit{d} mutant, synaptic transmission is not. There is an approximately fourfold decrease in the amplitude of evoked EJPs in the \textit{rl} \textit{d} mutant, which is very similar to the defect in evoked release observed in the \textit{unc-51} \textit{d} mutant. Remarkably, although both single mutants show large decreases in synaptic strength, evoked release returns to wild-type levels in
the double mutant. There is no significant difference in the amplitude of mEPJs in any of the four genotypes, so the change in evoked release is likely due to a change in the number of vesicles released from the presynaptic terminal. Calculating quantal content reveals that both the \textit{unc-51} mutant and \textit{rl}^1 release at least threefold fewer vesicles following an action potential, while release in the double mutant is indistinguishable from wild type (Fig. 8A–D). These findings have two implications. First, the suppression of the \textit{unc-51} phenotype by \textit{rl}^1 is consistent with the morphological findings and demonstrates that synaptic transmission in the \textit{unc-51} mutant is impaired due to the failure to inhibit ERK activity. Since increased ERK activity leads to a dra-
matic decrease in the number of structurally normal synapses, this is likely the cause of the defect in evoked release in the \textit{unc-51} mutant. Second, the deficit in evoked release in the \textit{rl1} mutant suggests that decreasing levels of ERK also disrupt evoked release, albeit in the absence of synaptic morphology defects, and that this can be suppressed by the loss of Unc-51 inhibition of ERK function. These results suggest that the Unc-51 kinase may bidirectionally regulate ERK activity to control synaptic strength.

Discussion

The close apposition of active zones to postsynaptic neurotransmitter receptors ensures that receptors encounter a high concentration of transmitter following synaptic vesicle fusion. However not every active zone formed by a single presynaptic cell has the same probability of releasing transmitter, implying that synapse-specific mechanisms exist to control active zone function. While many active zone proteins have been identified and characterized, little is known of the mechanisms that ensure that each active zone receives its proper complement of proteins. To identify such mechanisms, we have undertaken a genetic screen to identify genes required to ensure the apposition of such structurally normal active zones opposite each postsynaptic density. We demonstrate that the serine threonine kinase Unc-51 acts presynaptically to regulate the localization of the active zone protein Bruchpilot opposite to glutamate receptors at each synapse. In the absence of Unc-51, there is both a decrease in the density of synapses as well as the presence of abnormal synapses lacking their full complement of active zone proteins, leading to impaired transmitter release. Unc-51 regulates active zone development by inhibiting the activity of the MAP kinase ERK. This Unc-51-dependent inhibition of ERK activity provides a potential mechanism for synapse-specific control of active zone protein composition and release probability.

A screen for active zone mutants

Active zones are the site of synaptic vesicle docking and fusion (Rosenmund et al., 2003). Many active zone proteins have been identified (Zhai and Bellen, 2004; Zhen and Jin, 2004), although...
mutational analysis has not identified any proteins that are essential for active zone formation. Instead, loss of function analysis reveals defects in the function or morphology of active zones. For example, mutations in syd-2/liprin in both worms and flies lead to larger active zones, demonstrating its central role for organizing the active zone (Dai et al., 2006; Patel et al., 2006).

Drosophila contains orthologs of most vertebrate active zone proteins, the best characterized of which is Bruchpilot, the ortholog of CAST (Kittel et al., 2006; Wagh et al., 2006). Bruchpilot is present at the active zone, and promotes the active zone localization of calcium channels and the electron-dense T-bar specialization. As such, Bruchpilot is likely a key determinant of release probability at each active zone. Consistent with this view, synaptic transmission is impaired in bruchpilot mutants (Wagh et al., 2006), and active zones with a higher release probability tend to have more Bruchpilot (Marrus and DiAntonio, 2004).

We performed a large-scale anatomical screen to identify mutants where not every glutamate receptor cluster is apposed to Brp. We put aside mutants with a global decrease in Brp or DGluRIII across the NMJ, and instead focused on mutants in which Brp was absent from a subset of synapses. Such mutants were identified by the presence of glutamate receptor clusters unapposed to Bruchpilot puncta. In this screen we identified mutants in unc-51.

Unc-51 promotes the localization of Bruchpilot to every active zone
In the unc-51 mutant many DGluRIII clusters are unapposed to Brp. Such misapposition could reflect either DGluRIII clusters unapposed to active zones, or receptor clusters apposed to abnormal active zones that do not contain Brp. The ideal experiment to distinguish between these possibilities would be to stain for other presynaptic active zone proteins. Unfortunately the only other such protein that can be visualized in Drosophila is the calcium channel cacophony, and since its localization depends on Brp this experiment is not be informative. Nonetheless, two results strongly suggest that a subset of glutamate receptors is apposed to abnormal active zones. First, the decreased density of DGluRIII clusters observed via confocal microscopy approximates the decrease in active zone density observed via electron microscopy. If many DGluRIII clusters were unapposed to active zones, then we would have expected a more dramatic decrease in active zone density. Second, ultrastructural analysis demonstrates a decrease in the proportion of active zones containing T-bars. Brp is not necessary for the formation of active zones, but is required for the localization of T-bars to active zones (Kittel et al., 2006; Wagh et al., 2006). If the absence of Brp were due to the absence of the entire active zone, then each active zone would contain Brp and we would have predicted a normal ratio of T-bars/active zones.

Figure 8. Mutations in rolled suppress the unc-51 defect in evoked transmitter release. A, B, Representative spontaneous mEJPs (A) and EJPs (B) are shown from the same genotypes as in Figure 5. C, D, Histograms show the mean EJP amplitude (C) and quantal content (D) from WT (CS crossed to w−), unc-51 deletion mutant, homozygous rl1 hypomorphs, and rl1+unc-51 (rl1;unc-51). ***p < 0.001; n = 10 for all genotypes; and error bars represent SEM. p < 0.001 when either unc-51 or rl1 is compared with either wild type or double mutant. There is no significant difference between WT and double mutant (p = 0.3). Calibration: mEJP, 2.5 mV, 100 ms; EJP, 5 mV, 5 ms.
Instead, the decrease in T-bars/active zone is consistent with the presence of active zones missing Brp and, hence, lacking T-bars. Therefore, we conclude that Unc-51 is required for the high fidelity of active zone assembly, ensuring that Brp is present at every active zone.

In addition to the presence of abnormal synapses, the unc-51 mutant has, there is also a decrease in the number and density of synapses. We speculate that the decrease in synaptic density and the presence of abnormal synapses may be related phenotypes that differ in severity. In this view, Unc-51 promotes synapse formation. In its absence, active zone assembly would be less efficient, resulting in either the formation of abnormal active zones missing crucial proteins such as Brp, or in more severe cases leading to complete failure of active zone assembly and, hence, the absence of a synapse. The complete suppression of both the synaptic density and apposition phenotypes by mutation of the downstream target ERK is consistent with these phenotypes sharing an underlying mechanism. As expected, this defect in the number and proper assembly of synapses leads to a dramatic decrease in synaptic efficacy.

In addition to these synaptic defects, the unc-51 mutant also has a smaller NMJ and accumulations of synaptic material in the axons, suggesting defects in axonal transport. One mechanism that could link a small NMJ with defective transport is synaptic retraction, in which entire presynaptic boutons or branches retract leaving a footprint of postsynaptic proteins (Eaton et al., 2002). However, we did not observe such footprints in the unc-51 mutant, so this is not the cause of the small NMJ. Synaptic growth requires the retrograde transport of a BMP signal to the nucleus (Marquès et al., 2003; McCabe et al., 2003), however we found no change in the levels of phosphorylated MAD in motoneuron nuclei (data not shown), suggesting that this is not a likely cause of the growth defect. Finally, in worms and mice Unc-51 is required for axon outgrowth, which may be somewhat analogous to defects in NMJ growth in Drosophila. However, to form an NMJ the axon must navigate out of the ventral nerve cord and cross a wide expanse of muscle before reaching its target and forming a junction. Since we do not observe defects in the pattern of neuromuscular innervation, it is unlikely that a generic defect in axon outgrowth is responsible for the small NMJs. The apparent axonal transport defect is consistent with findings from mammals suggesting a function for Unc-51 in regulating axon transport. We have not investigated the role of Unc-51 for transport, but note that we were able to genetically separate the axonal transport and synapse development phenotypes, so the transport phenotypes may not be primary cause of the synaptic defects.

Unc-51 inhibits ERK signaling to promote synapse formation

Our data support the model that Unc-51 inhibits ERK activation to promote proper active zone development. In the unc-51 mutant we observed a modest increase in the levels of activated ERK, to promote proper active zone development. In the BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167:761–781.

References

Regulating release probability at single release sites

In mammalian and Drosophila neurons, release probability varies across release sites formed by a single neuron (Rozov et al., 2001; Marrus and DiAntonio, 2004). One potential mechanism would be the differential localization or activity of core active zone proteins. In Drosophila, Bruchpilot is an excellent candidate for such a protein. It is required for the localization of calcium channels to the active zone, so changes in its localization or function would impact calcium influx and, hence, release probability at an active zone. The unc-51 mutant demonstrates that signaling pathways can differentially regulate the localization of Brp to individual release sites within a single neuron. As such, the Unc-51/Erk signaling pathway is a candidate mechanism to regulate active zone protein composition and release probability in a synapse-specific manner.

Unc-51 inhibits ERK signaling to promote synapse formation

Our data support the model that Unc-51 inhibits ERK activation to promote proper active zone development. In the unc-51 mutant we observed a modest increase in the levels of activated ERK, demonstrating that Unc-51 is a negative regulator of ERK activation in vivo. This increased ERK activity is responsible for the defects in active zone formation. Double mutants between unc-51 and the ERK hypomorph rl1 completely suppress the synaptic density and apposition phenotypes of the unc-51 mutant, and restore synaptic strength to wild-type levels. Hence, ERK is required for the synaptic phenotypes observed in the unc-51 mutant. The axonal transport defects were not suppressed in the double mutant, so Unc-51 must act through other pathways as well. In mammalian cells Unc-51 can downregulate ERK by inhibiting the binding of a scaffolding protein to the FGF receptor. To date, no receptor tyrosine kinase has been identified that regulates active zone formation in Drosophila. Future studies to characterize the mechanism by which Unc-51 inhibits ERK in Drosophila motoneurons may provide clues toward identification of such a pathway. In addition, it is unclear how ERK regulates active zone formation. A previous study demonstrated that phospho-ERK localizes to the active zone, which would suggest a direct mechanism (Koh et al., 2002). Unfortunately, we have been unable to replicate these localization findings. The same study demonstrated that the transgenic expression of a constitutively active ras or a gain-of-function ERK allele both lead to an increase in the number of synaptic boutons, which is not consistent with our finding of a smaller NMJ. Active zone structure and number were not assessed. We speculate that the global activation of ERK may result in different phenotypes than relief of Unc-51 inhibition of ERK, which could show temporal and spatial specificity.

References


