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The Notch ligand E3 ligase, Mind Bomb1, regulates glutamate receptor localization in *Drosophila*

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Abstract

The postsynaptic density (PSD) is a protein-rich network important for the localization of postsynaptic glutamate receptors (GluRs) and for signaling downstream of these receptors. Although hundreds of PSD proteins have been identified, many are functionally uncharacterized. We conducted a reverse genetic screen for mutations that affected GluR localization using *Drosophila* genes that encode homologs of mammalian PSD proteins. 42.8% of the mutants analyzed exhibited a significant change in GluR localization at the third instar larval neuromuscular junction (NMJ), a model synapse that expresses homologs of AMPA receptors. We identified the E3 ubiquitin ligase, Mib1, which promotes Notch signaling, as a regulator of synaptic GluR localization. Mib1 positively regulates the localization of the GluR subunits GluRIIA, GluRIIB, and GluRIIC. Mutations in *mib1* and ubiquitous expression of Mib1 that lacks its ubiquitin ligase activity result in the loss of synaptic GluRIIA-containing receptors. In contrast, overexpression of Mib1 in all tissues increases postsynaptic levels of GluRIIA. Cellular levels of Mib1 are also important for the structure of the presynaptic motor neuron. While deficient Mib1 signaling leads to overgrowth of the NMJ, ubiquitous overexpression of Mib1 results in a reduction in the number of presynaptic motor neuron boutons and branches. These synaptic changes may be secondary to attenuated glutamate release from the presynaptic motor neuron in *mib1* mutants as *mib1* mutants exhibit significant reductions in the vesicle-associated protein cysteine string protein and in the frequency of spontaneous neurotransmission.

Keywords

Postsynaptic Density; Glutamate Receptors; Synapse; Synaptic Transmission; *Drosophila* Neuromuscular Junction

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Introduction

Proper formation and maintenance of glutamatergic synapses is required for diverse neurobiological processes including movement (Girault, 2012), visual processing (Self *et al.*, 2012), and learning and memory (Hu *et al.*, 2007; Matsuo *et al.*, 2008; Sanderson & Bannerman, 2012). Once established, these synapses are plastic and modify themselves as a result of changes in activity. Synaptic plasticity occurs as a result of changes in presynaptic neurotransmitter release probability, the localization and synthesis of synaptic proteins, and remodeling of the synaptic cytoskeleton (reviewed in (Huganir & Nicoll, 2013; Padamsey & Emptage, 2014)). The localization of postsynaptic ionotropic glutamate receptors (GluRs) opposite of presynaptic release sites is particularly important for synaptic transmission as it determines the postsynaptic response (Xie *et al.*, 1997; DiAntonio *et al.*, 1999; Franks *et al.*, 2003; Raghavachari & Lisman, 2004; Lisman *et al.*, 2007).

Excitatory postsynaptic GluRs are components of the postsynaptic density (PSD), a specialized network of proteins that links receptors to the cytoskeleton and downstream signaling pathways. The PSD, localized to mammalian small postsynaptic protrusions or dendritic spines, is estimated to contain hundreds of different proteins (Satoh *et al.*, 2002; Jordan *et al.*, 2004; Peng *et al.*, 2004; Yoshimura *et al.*, 2004; Collins *et al.*, 2006; Dosemeci *et al.*, 2006; Bayes *et al.*, 2011), many of which are represented by multiple copy numbers (Chen *et al.*, 2008; Shinohara, 2011). PSD proteins can be broadly grouped as cell adhesion molecules, cytoskeletal proteins, metabolic proteins, transmembrane proteins, trafficking/motor proteins, scaffold proteins, and enzymes like GTPases and kinases/phosphatases (Okabe, 2007). In mammals, dysfunction of the PSD is linked to neurodegenerative diseases (for review see (Gong & Lippa, 2010)), autism/autism spectral disorders (Feyder *et al.*, 2010; Bangash *et al.*, 2011), schizophrenia (Hashimoto *et al.*, 2007; Cheng *et al.*, 2010), mental impairments (Raymond & Tarpey, 2006; Zanni *et al.*, 2010), and drug abuse (Moron *et al.*, 2007; Okvist *et al.*, 2011).

The composition and size of the PSD are dynamically regulated by synaptic activity. Long-term potentiation (LTP), a process that enhances synaptic efficacy and is thought to be the cellular basis of learning and memory (Neves *et al.*, 2008; Takeuchi *et al.*, 2014), results in the redistribution of the AMPA receptor subunit, GluA1, and NMDA receptor subunit, GluN1, to dendritic areas of the rat dentate gyrus (Kennard *et al.*, 2014). The increased surface localization of GluA1 is mediated by remodeling of the actin cytoskeleton (Gu *et al.*, 2010; Kerr & Blanpied, 2012) and may be linked to altered localization of scaffolding proteins within the PSD (MacGillavry *et al.*, 2013; Bosch *et al.*, 2014; Meyer *et al.*, 2014). LTP also results in expansion of the PSD (Chen *et al.*, 2007; Bosch *et al.*, 2014) and enlargement of dendritic spines (Matsuzaki *et al.*, 2004; Harvey & Svoboda, 2007), both of which require local translation of PSD components (Bramham, 2008; Bosch *et al.*, 2014).

PSD proteins are remarkably conserved with orthologs across archaeal, bacteria, and eukaryote kingdoms (Emes *et al.*, 2008; Alie & Manuel, 2010; Emes & Grant, 2011). We have previously identified *Drosophila* orthologs for approximately 96% of published mammalian PSD proteins (Liebl & Featherstone, 2008). The functional role of many of these proteins is currently unidentified in any species. Therefore, we performed a reverse

genetic screen to determine whether mutations in *Drosophila* PSD orthologs affect the synaptic localization of GluRs at the neuromuscular junction (NMJ) using immunocytochemistry. The *Drosophila* larval NMJ contains ionotropic GluRs that are homologous to AMPA receptors (Menon *et al.*, 2013). We uncovered a novel function for the E3 ubiquitin ligase, Mind Bomb1 (Mib1), a component of the Notch signaling pathway, in the regulation of postsynaptic GluR localization. Mib1 regulates the clustering of postsynaptic GluRs, the frequency of spontaneous neurotransmission, and synaptic levels of the presynaptic protein cysteine string protein (CSP).

Results

Reverse genetic screen for gene products that regulate GluR localization

The PSD is a dense protein network opposed to presynaptic release sites that helps provide the structural basis for synaptic regulation and plasticity (Collins *et al.*, 2006; Dosemeci *et al.*, 2006). Hundreds of PSD proteins have been identified and the *Drosophila* genome encodes orthologs for 95.8% of these proteins (Liebl & Featherstone, 2008). Many of these genes are functionally uncharacterized. Therefore, we conducted a reverse genetic screen of genes that encode homologs of mammalian PSD proteins to identify mutants with altered postsynaptic GluR expression and/or localization at the 6/7 NMJ of third instar *Drosophila* larvae. This NMJ is innervated by two glutamatergic motor neurons that arborize on muscles by forming a series of distinct swellings or boutons (Jan & Jan, 1976; Johansen *et al.*, 1989; Ruiz-Canada & Budnik, 2006). *Drosophila* NMJ GluRs are similar to non-NMDA receptors including AMPA receptors and are tetramers that contain either the GluRIIA or GluRIIB subunits along with GluRIIC (Marrus *et al.*, 2004), GluRIID (Featherstone *et al.*, 2005), and GluRIIE (Qin *et al.*, 2005).

We examined 130 different mutations that corresponded to 144 mammalian PSD proteins (Table S2) for altered synaptic localization of the GluRIIA subunit. 18 mutations (12.5%) were lethal prior to the third instar larval stage and, therefore, were not analyzed. Of the remaining mutants analyzed, 48 (42.8%) exhibited phenotypes that significantly affected the localization of postsynaptic GluRs as indicated by a significant change in relative GluRIIA fluorescence intensity. The majority of these mutations (42/48 or 87.5%) resulted in a significant reduction in postsynaptic GluRs containing GluRIIA. Conversely, six mutations (6/48 or 12.5%) produced an increase in synaptic GluRIIA.

We found that mutations in genes encoding cell adhesion molecules, cytoskeletal proteins, metabolic proteins, transmembrane proteins, trafficking/motor proteins, scaffold proteins, and enzymes led to significant changes in GluRIIA synaptic fluorescence (Tables 1, S2). To further explore these synaptic phenotypes, subsets of mutants were examined for changes in GluRIIA cluster sizes. Postsynaptic GluRIIA-containing receptors localize in clusters or puncta in apposition to presynaptic active zones (Petersen *et al.*, 1997), sites of neurotransmitter release. The size and intensity of these clusters parallels the function of the synapse (Featherstone *et al.*, 2002). Although GluRIIA cluster sizes correlated with relative GluRIIA fluorescence intensity in the mutants identified in the screeen (Figs. 1A–B, 2A–B), there were not consistent changes observed in the morphology of the presynaptic motor neuron (Figs. 1C, 2C).

Mib1 positively regulates GluR clustering

One mutation that led to a reduction in synaptic GluRIIA was in *mind bomb1* (*mib1*), which was also identified in a similar forward genetic screen in our lab. *Drosophila* Mib1 is 66.6% identical and 76.9% similar to human Mib1 (http://blast.ncbi.nlm.nih.gov/ using NP_678826.2 and NP_065825.1 accession numbers, respectively). Mib1 is an E3 ubiquitin ligase localized to the PSD (Choe *et al.*, 2007) that promotes Notch signaling by regulating endocytosis of the Notch ligands Delta (Koo *et al.*, 2005a) and Jagged/Serrate (Lai *et al.*, 2005; Le Borgne *et al.*, 2005; Koo *et al.*, 2007). Although Mib1 is important for neuronal differentiation in both the central (Haddon *et al.*, 1998; Ossipova *et al.*, 2009; Yamamoto *et al.*, 2010) and peripheral (Kang *et al.*, 2013) nervous systems, we did not observe differences in the sizes of the ventral nerve cord or muscles in *mib1* mutants (data not shown). Similarly, there were no significant differences in synaptic or muscle acetylated tubulin levels or the sarcomeric structure of the muscle as indicated by phalloidin labeling in *mib1* mutants (data not shown). Therefore, we sought to characterize the role of Mib1 in GluR localization.

Two mutant alleles were employed to assess the synaptic role of Mib1 including $mib1^{EY09780}$, which contains a transposable element in the 5' end of the *mib1* coding sequence, and $mib1^{3}$, which is a null mutation that introduces an early stop codon (Le Borgne *et al.*, 2005). The latter causes early larval lethality. Therefore, $mib1^{3}$/ $mib1^{EY09780}$ transheterozygous mutants were used in our experiments. Both $mib1^{EY09780}$ and $mib1^{3}$/ $mib1^{EY09780}$ mutants exhibited a significant reduction in GluRIIA cluster sizes compared with controls (Fig. 3A–B). The reduction in cluster sizes corresponded to a reduction in relative GluRIIA fluorescence intensity in both mutant genotypes but this was not significant. Although there were slight, consistent increases in the number of motor neuron branches and boutons, these increases were not significant (Fig. 3C). Similar to GluRIIA, there were significant reductions in GluRIIB (Fig. 4A–B) and GluRIIC (Fig. 4C–D) cluster sizes in $mib1^{EY09780}$ and $mib1^{3}$/ $mib1^{EY09780}$ mutants and this corresponded to a significant reduction in relative fluorescence for each subunit.

Notch signaling is initiated by Notch binding to its ligand on adjacent cell surfaces. This leads to the proteolytic cleavage of Notch at two sites (van Tetering & Vooijs, 2011) and endocytosis of both the Notch intracellular domain and the ligand in the adjacent cell (Chitnis, 2006; Brou, 2009). The intracellular domain of Notch translocates into the nucleus and binds to transcription factors of the CBF1/Su(H)/Lag1 (CSL) family thereby activating transcription of hundreds of target genes (Borggrefe & Liefke, 2012). To investigate the possibility that Mib1 may influence *GluR* transcript levels by regulating the transcriptional activity of the Notch signaling pathway, we assessed relative mRNA levels using qRT-PCR. *GlurIIA*, *glurIIB*, and *glurIIC* transcript levels were not significantly altered in *mib1* mutants (Fig. 4E). These data indicate that Mib1 likely regulates the localization or postsynaptic stabilization of GluRs but does not affect transcription of *glur* subunits.

Overexpression of mib1 increases GluR cluster sizes while deleting the mib1 ring finger domain decreases GluR cluster sizes

To confirm the role of Mib1 in GluR clustering, we first expressed a *mib1* transgene that lacks the region encoding the C-terminal RING finger domains (*UAS-mib1*$^{\Delta 3RF}$). Mib1 contains three RING finger domains (Itoh *et al.*, 2003) that mediate ubiquitination of the Notch ligands Delta (Chen & Casey Corliss, 2004) and Serrate (Lai *et al.*, 2005). Mib1Δ3RF interacts with Delta and Serrate but does not endocytose these ligands thereby inhibiting Notch signaling (Lai *et al.*, 2005). Similar to *mib1* mutants, expression of the *UAS-mib1*$^{\Delta 3RF}$ transgene in all cells using the *Actin5c-Gal4* driver resulted in a significant reduction in GluRIIA cluster sizes and relative fluorescence (Fig. 5A–B) compared with outcrossed controls. Although there was a significant increase in branching of the presynaptic motor neuron in animals expressing *mib1*$^{\Delta 3RF}$ in all tissues compared with outcrossed controls, there was no significant change in the number of boutons (Fig. 5C).

Next, we overexpressed wild type Mib1 using a transgene previously shown to enhance Notch signaling (Lai *et al.*, 2005). There were significant increases in both GluRIIA cluster sizes and relative fluorescence in animals overexpressing *mib1* in all tissues compared with outcrossed controls (Fig. 6A–B). Overexpression of *mib1* also led to a reduction in the size of the presynaptic motor neuron as indicated by a significant decrease in motor neuron boutons and branches (Fig. 6C). These results collectively suggest that Notch signaling positively correlates with GluR levels at the synapse but negatively correlates with the size of the presynaptic motor neuron.

Mib1 Regulates Synaptic Levels of CSP and FasII

We next examined the levels of several synaptic proteins to determine if Mib1 may influence the localization of other proteins important for synaptic function. Mutations in *mib1* did not affect the density of Bruchpilot (Brp; Fig. 7A), which is localized to presynaptic active zones where it helps to organize Ca^{2+} channels (Kittel *et al.*, 2006; Wagh *et al.*, 2006) and synaptic vesicles (Matkovic *et al.*, 2013). Similarly, the loss of Mib1 did not affect synaptic levels of the scaffold protein discs large (DLG, Fig. 7C), which acts as an adaptor protein required for GluRIIB clustering in embryos (Chen & Featherstone, 2005). Both cysteine string protein (CSP) and Fasciclin II (FasII) levels, however, were significantly affected in *mib1*^{3}/ *mib1*^{EY09780} mutants. CSP, a vesicle-associated protein important for evoked neurotransmitter release (Bronk *et al.*, 2005) and presynaptic protein folding (Donnelier & Braun, 2014), was significantly reduced at *mib1*^{3}/ *mib1*^{EY09780} mutant NMJs (Fig. 7B). Although there was a slight reduction in CSP levels in *mib1*^{EY09780} mutants, it was not significant. Conversely, *mib1*^{3}/ *mib1*^{EY09780} but not *mib1*^{EY09780} mutants exhibited a significant increase in synaptic levels of the homophilic cell adhesion molecule FasII (Fig. 7D), which regulates synaptic growth (Schuster *et al.*, 1996) and postsynaptic organization during synaptogenesis (Kohsaka *et al.*, 2007). Collectively, these data indicate that, in addition to GluRs, Mib1 is important for the synaptic localization of CSP and FasII.

Mib1 regulates the frequency of spontaneous neurotransmission

The change in the synaptic localization of GluRs, CSP, and FasII could affect the function of the synapse. Therefore, we assessed the function of *mib1* mutant NMJs using two-electrode

voltage clamp electrophysiology. Both *mib1*^{EY09780} and *mib1*^{3}/ *mib1*^{EY09780} mutants exhibited a significant decrease in the frequency of miniature endplate junctional currents (mEJCs) but not in mEJC amplitudes compared with controls (Fig. 8). Although *mib1* mutants also exhibited decreased evoked endplate junctional current (eEJC) amplitudes and quantal content, these reductions were not significant. Based on these data, we conclude that the function of *mib1* mutant NMJs is affected likely as a result of altered localization of synaptic proteins.

Discussion

The functions of many PSD proteins are poorly characterized. To better understand the relationship between PSD components and the localization of postsynaptic GluRs, we conducted a reverse genetic screen to identify mutations that affected the synaptic localization of GluRs. *Drosophila* orthologs were previously identified for 95.8% of genes that encode mammalian PSD proteins (Liebl & Featherstone, 2008) and mutations in 130 of these orthologs were examined here. We next focused on one ortholog, *mib1*, to better understand how it influences the structure and function of synapses.

GluRs are shuttled in and out of the synaptic membrane as a result of changes in synaptic activity ultimately altering the strength of the synapse (Chater & Goda, 2014; Shipton & Paulsen, 2014; Sihra *et al.*, 2014). The synaptic localization of GluRs is directly mediated by components of the PSD including transmembrane proteins associated with GluRs and scaffolding proteins (Jackson & Nicoll, 2011; Verpelli *et al.*, 2012). Other components of the PSD including the actin cytoskeleton and many enzymes that regulate protein interactions influence the localization of GluRs without directly binding to them (Okabe, 2007). 42.8% of the mutations we examined showed significant changes in synaptic GluRIIA localization (Table S2). Given the role of the PSD in GluR localization, we might expect that percentage to be higher. It is important to note, however, that we did not examine GluRIIB localization in these same mutants. The GluRIIA and GluRIIB subunits are mutually exclusive in the GluR tetramer (Marrus *et al.*, 2004; Featherstone *et al.*, 2005; Qin *et al.*, 2005), differentially localized to the synapse (Marrus *et al.*, 2004; Schmid *et al.*, 2008), and are stabilized by unique PSD proteins (Chen & Featherstone, 2005; Chen *et al.*, 2005). Thus, some mutations could affect the localization of GluRIIB without also affecting GluRIIA. In addition, we used *P*-element mutants in our screen. Because these transposon insertions typically result in hypomorphic mutations (Spradling *et al.*, 1995; LaFave & Sekelsky, 2011), we may have missed phenotypes that would have resulted from the use of null alleles.

Most mutations that affected GluR localization resulted in the loss of synaptic GluRIIA and this is consistent with previous screens (Liebl & Featherstone, 2005). These data suggest that most regulatory proteins promote the trafficking and localization of GluRIIA-containing receptors. Indeed, the PSD proteins KRIP6 and S-SCAM have been shown to be important for the membrane localization of the Kainate receptor subunit GluR6 and the AMPA receptor subunit GluA2 (Laezza *et al.*, 2008; Danielson *et al.*, 2012). Mutations in the *Drosophila* orthologs *diablo* (*dbo*) and *magi* produce a significant reduction in the synaptic localization of GluRIIA (Fig. 1). The loss of GluRIIA in *moesin* (*moe*) mutants may be due

to the proposed role of Moe in binding and stabilizing the actin cytoskeleton. Moe and radixin are important for the actin-dependent processes of growth cone extension in rat cultured neurons (Paglini *et al.*, 1998) and Moe is localized specifically to polymerized actin at the *Drosophila* NMJ (Khuong *et al.*, 2010).

Half of the mutations that produced an increase in synaptic GluRIIA were in genes encoding proteins and GTPases important for remodeling of the actin cytoskeleton. Sra-1 interacts with Abelson interacting protein (Abi-1) (Steffen *et al.*, 2004), which activates WAVE2 to promote actin nucleation (Leng *et al.*, 2005) in non-neuronal cells. Similarly, the Rho family GTPase, Rac2, enhances actin nucleation by activating cofilin (Sun *et al.*, 2007). Remodeling of the actin cytoskeleton is essential for structural changes to the dendritic spine (Fortin *et al.*, 2012) and the stabilization of newly incorporated AMPA receptors (Rudy, 2014). Mutations in *abi*, *rac2*, and *sra-1* may inhibit actin dynamics such that GluRs are retained in the synapse as we observed (Fig. 2). In support of this, several proteins required for clathrin-dependent endocytosis interact with WASP, WAVE, and Cdc42, proteins that enhance nucleation of the actin cytoskeleton (Saheki & De Camilli, 2012). AMPA receptors are endocytosed during long-term depression (LTD) by clathrin-dependent endocytosis (Anggono & Huganir, 2012; Hanley, 2014).

The Notch signaling protein, Mib1, was one of 42 mutations that resulted in a significant decrease in synaptic levels of GluRIIA. The importance of Notch signaling in cell fate determination is well established in many cell types including neurons (Louvi & Artavanis-Tsakonas, 2006). More recent studies have identified roles for Notch in cell division, axon guidance and synaptogenesis (Giniger, 2012). We sought to better characterize the role of Mib1, which promotes Notch signaling by regulating the endocytosis of the Notch ligands Delta (Koo *et al.*, 2005a) and Jagged/Serrate (Lai *et al.*, 2005; Le Borgne *et al.*, 2005; Koo *et al.*, 2007), in terminally differentiated neurons. We found that, in addition to GluRIIA, Mib1 positively regulates the localization of the GluR subunits GluRIIB and GluIIC. Our data indicate that attenuation of Notch signaling by expressing a ligase-deficient Mib1 (Mib1Δ3RF; (Lai *et al.*, 2005)) in all tissues (Fig. 5) or as a result of mutations in *mib1* (Figs. 3–4) produces a loss of synaptic GluRs. Further, mutations in *polychaetoid* (*pyd*), which promotes Notch signaling in sensory organ precursors (Chen *et al.*, 1996), significantly reduced synaptic GluRIIA levels (Fig. 1). Conversely, enhanced Notch signaling as a result of overexpressing Mib1 in all tissues (Lai *et al.*, 2005) led to increased synaptic levels of GluRIIA (Fig. 6).

One potentially confounding variable exists in interpreting our overexpression data. Ubiquitous expression of Mib1 could lead to misexpression phenotypes as Mib1 may only be localized to one cell type at the NMJ. Mib1 is expressed in cells containing Notch ligands, which are localized adjacent to cells that express the Notch receptors (Itoh *et al.*, 2003). Notch receptors were previously detected in presynaptic motor neuron cell bodies at the Drosophila NMJ (de Bivort *et al.*, 2009). Expression of Mib1Δ3RF in all tissues, however, would only affect the phenotype of Mib1-expressing cells because the RING finger domains are required Mib1 ubiquitin ligase activity (Lai *et al.*, 2005). The similarity of the phenotypes in mutants deficient in Notch signaling strongly suggests that Notch signaling and Mib1 regulate the localization of GluRs at the NMJ.

This is the first report to show that Notch signaling alters the synaptic levels of glutamate receptors. Although conditional knockout of *mib1* impaired memory for hippocampal-dependent tasks and attenuated late LTP and LTD, it did not alter synaptic levels of several GluR subunits including GluA1, GluA2/3, GluN1, GluN2A, or GluN2B (Yoon *et al.*, 2012). Conversely, *mib1* mutant zebrafish showed significant reductions in GluR subunit mRNAs for *AMPA 2a* and *AMPA 2b* and the glutamate metabolizing gene product, *glutamate decarboxylase*, as indicated by microarray analyses (Hortopan *et al.*, 2010). Our data suggest that Mib1 likely regulates GluR subunits posttranscriptionally as we did not detect appreciable differences in *glur* mRNA levels in *mib1* mutants (Fig. 4E).

Mib1 may directly regulate the localization of GluRs. AMPA receptor subunits localized to the cell membrane are ubiquitinated after enhanced synaptic activity (Widagdo *et al.*, 2015). The Mib1 paralog, Mib2 (Koo *et al.*, 2005b), directly binds and ubiquitinates the GluN2B but not the GluN1 subunit of the NMDA receptor in an activity-dependent manner. This ultimately decreased NMDA-mediated synaptic currents (Jurd *et al.*, 2008). Our data, however, suggest that Mib1 indirectly regulates the localization of GluR subunits. If Mib1 functioned similar to Mib2 to directly regulate the localization of non-NMDA receptors, we would expect to see an increase in GluR subunits in *mib1* mutants and after ubiquitous expression of Mib1Δ3RF. Instead we observe a reduction in synaptic levels of GluRIIA (Fig. 3), GluRIIB, and GluRIIC (Fig. 4) in *mib1* mutants. Therefore, we favor the hypothesis that Mib1 attenuates the presynaptic release of glutamate, which, over developmental time, leads to a reduction in synaptic GluR subunit levels.

In support of this hypothesis, we observe a significant reduction in CSP at *mib1* mutant NMJs (Fig. 7B). Mouse *CSP*-α knock out NMJs exhibit deficient presynaptic vesicle endocytosis and a reduction in the size of the readily releasable pool followed by reduced synaptic vesicle exocytosis (Rozas *et al.*, 2012). Similarly, we observe a significant reduction in mEJC frequency in *mib1* mutants (Fig. 8C) indicating a reduction in presynaptic glutamate release. Although it may seem counterintuitive that loss of a PSD protein could result in altered levels of CSP, which is primarly localized presynaptically (Kohan *et al.*, 1995), and neurotransmitter release, Mib1 activates Notch signaling in neighboring cells (Koo *et al.*, 2005a; Lai *et al.*, 2005; Le Borgne *et al.*, 2005; Koo *et al.*, 2007). Thus, Mib1 localized to the PSD would activate Notch signaling in the adjacent presynaptic cell. Altered presynaptic Notch signaling resulting from loss of Mib1 activity, could then affect the expression of Notch target genes thereby affecting cellular function.

In summary, we have found that mutations in several genes that encode orthologs of mammalian PSD proteins are important for the proper localization of GluRs at the *Drosophila* NMJ. The PSD protein, Mib1, positively regulates the synaptic localization of GluRIIA, GluRIIB, and GluRIIC. The localization of GluRs may be secondary to Mib1's role in localizing presynaptic CSP and regulating the spontaneous release of neurotransmitter.

Experimental Methods

Fly Stocks

Fly stocks were raised at 25°C on Jazz Mix food (Fisher Scientific, St. Louis, MO). *Drosophila* orthologs and corresponding mammalian PSD proteins were previously identified (Liebl & Featherstone, 2008). Mutant stocks for the reverse genetic screen were identified using FlyBase (http://flybase.org/) and obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/). Identities of the stocks can be found in Table S2. Most of the lines used (65.9%) were homozygous adult viable. However, the remaining lines (34.1%) were homozygous adult lethal and balanced using chromosome-specific GFP-balancers or the TM6 Tb balancer to enable identification of homozygous mutants. Both $mib1^{3}$ and $mib1^{EY09780}$ alleles and all *Gal4* drivers were obtained from the Bloomington *Drosophila* Stock Center. Stocks containing the *UAS-*$mib1^{\Delta 3RF}$ and *UAS-*$mib1^{wt}$ transgenes were generous gifts from Eric Lai (Lai *et al.*, 2005). Control stocks included w^{1118} and w^{1118} outcrossed to *Actin5c-Gal4, 24B-Gal4, elav-Gal4, UAS-*$mib1^{\Delta 3RF}$, and *UAS-*$mib1^{wt}$.

Immunocytochemistry and Confocal Microscopy

Third instar larvae were filet dissected on Sylgard-containing petri dishes at room temperature in Roger's Ringer solution (135 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM TES, 72 mM sucrose) supplemented with 2 mM glutamate (Augustin *et al.*, 2007). Larval preparations were fixed for 30 min with either Bouin's fixative (Fisher Scientific, St. Louis, MO) for Brp or GluR antibodies or 4% paraformaldehyde in PBS for all other antibodies. Primary antibodies were diluted in PBTX (PBS + 0.1% Triton and 1% Bovine Serum Albumin) and applied overnight at 4°C after larvae were washed PTX (PBS + 1% Triton). Mouse α-Brp (aka nc82, 1:50), mouse α-CSP (1:200), mouse α-DLG (1:1000), mouse α-FasII (1:5), and mouse α-GluRIIA (1:100) were obtained from the Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit α-GluRIIB (1:2000) and rabbit α-GluRIIC (1:5000) were generous gifts from Aaron DiAntonio (Marrus *et al.*, 2004). Mouse α-acetylated tubulin and phalloidin (1:200) were obtained from Sigma Aldrich (Cat #, St. Louis, MO) and Invitrogen (Cat #, Carlsbad, CA), respectively. Additional antibodies including HRP (1:125) and species-specific FITC (1:250) were obtained from Jackson Immunolabs (West Grove, PA), diluted in PBTX, and applied for 2 h at room temperature. Larval preparations were mounted on slides and covered with Vectashield (Vector Labs, Burlingame, CA).

Larval A3 or A4 6/7 NMJ were imaged using a Fluoview 1000 Olympus confocal laser scanning microscope. Imaging parameters were set for controls and subsequently used for all experimental animals. Equal numbers of control and experimental animals were imaged each day. Compressed images of the z-series were used for data analyses.

Electrophysiology

Third instar larvae were filet dissected and secured using VetBond glue (World Precision Instruments, Sarasota, FL) at room temperature in Roger's Ringer on Sylgard-coated coverslips. Two-electrode voltage clamp recordings were obtained from muscle 6 of A3 or

A4 after clamping the muscle membrane potential at −60 mV using an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA). Both the clamp and recording electrodes were filled with 3 M KCl and used if their resistances were 10–20 MΩ. The stimulating electrode was filled with bath saline. For evoked recordings, a 1 Hz, 10V stimulus was delivered by a Grass S88 stimulator with a SIU5 isolation unit (Grass Technologies, Warwick, RI) to recruit both motor neuron axons as previously described (Ehmann *et al.*, 2014). Recordings were digitized with a Digidata 1443 digitizer (Molecular Devices, Sunnyvale, CA). PClamp software (v. 10.4) was used for data analyses. Quantal content was calculated by dividing the eEJC area (nA*ms) by the mEJC area (nA*ms).

qRT-PCR

RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) from 8–12 third instar larvae as previously described (Jowett, 1998). qRT-PCR was performed in single-plex reactions using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA), gene-specific primers for *gluRIIA*, *gluRIIB*, *gluRIIC*, and *GAPDH*, and the Stratagene Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA). 100 ng of total RNA was added to each reaction. Three technical replicates and two biological replicates were performed for each reaction. Relative *gluR* mRNA levels were obtained by subtracting the *GAPDH* C(t) value from the *GluR* C(t) value.

Data Analysis and Statistics

The number of boutons and branches were obtained by manually counting these features using 6/7 NMJs of hemisegments A3 or A4. Branches were defined as an extension of the presynaptic motor neuron with more than one bouton. The density of Brp labeling was quantified by counting the total number of Brp puncta and dividing by the total NMJ area as indicated by HRP labeling using ImageJ (NIH) software. GluR cluster sizes were measured by manually tracing around the GluR puncta overlapping and immediately adjacent to HRP immunloabeling and measuring the area with Image J software as previously described (Featherstone *et al.*, 2002). For all other immunolabeling, immunoreactivity was quantified by measuring the mean fluorescence intensity of the NMJ using Adobe Photoshop software (v. CS2) and subtracting the mean non-NMJ background over an identical area of the neighboring muscle membrane. For DLG and muscle acetylated tubulin, the average background from a non-synaptic, non-muscle area was used. 8–12 animals per genotype were used for analyses for the reverse genetic screen.

Data analyses were conducted with GraphPad Prism (v5.01). Student’s t-tests were used for experiments with a single control. For experiments with more than one control, an ANOVA was performed with a Tukey post hoc test. In figures, $p<0.0001$ is designated by ***, $p<0.001$ is designated by **, and $p<0.05$ is designated by *. Error bars are representative of standard error of the mean values. Summary statistics for all data are reported in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Identified *Drosophila* PSD homologs important for glutamate receptor localization.
- Further characterized the Notch signaling protein, Mib1.
- Mib1 positively regulates glutamate receptor localization at the *Drosophila* NMJ.
- Mib1 may secondarily affect glutamate receptors by influencing glutamate release.

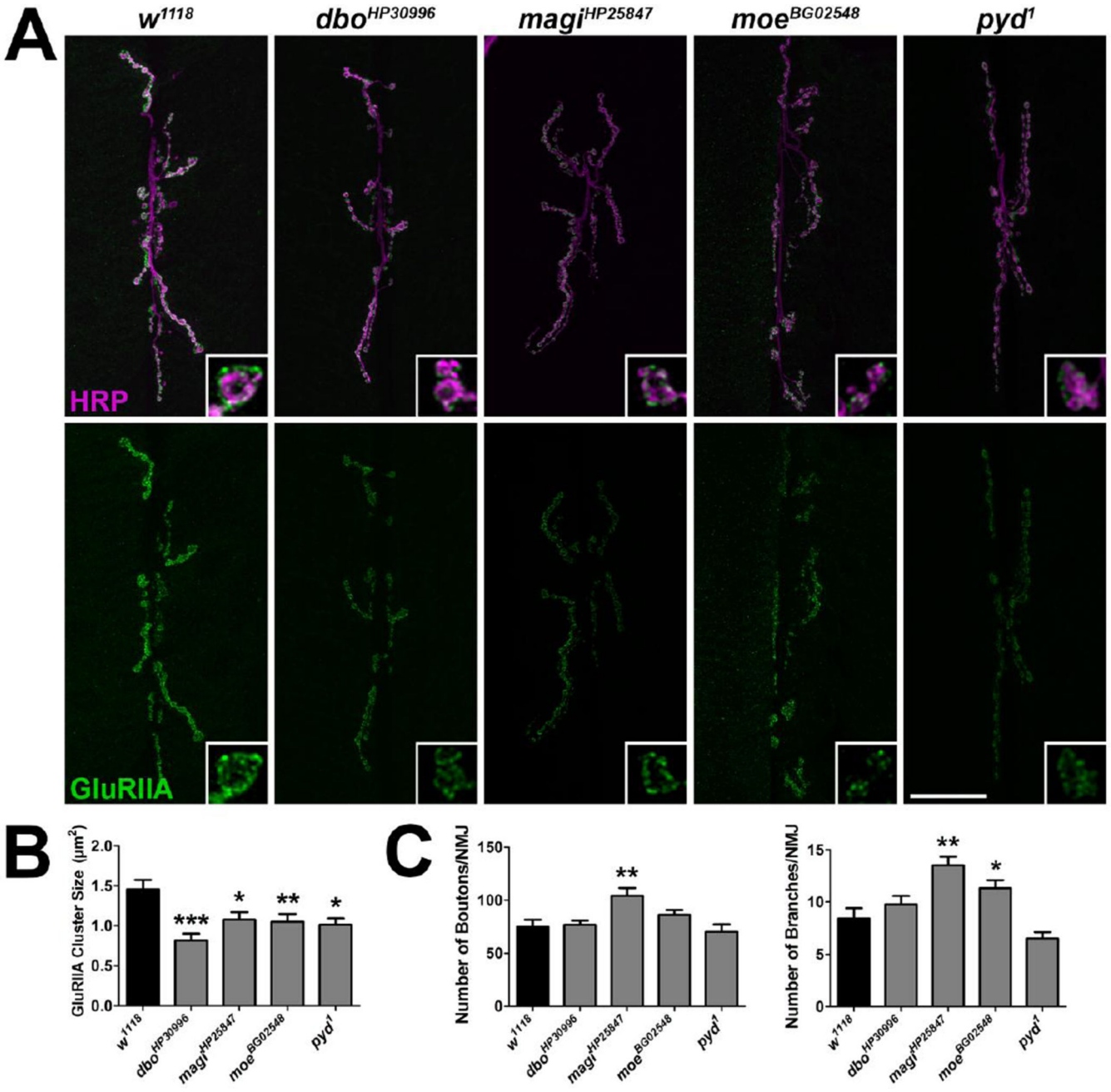

Figure 1. Mutations in *Drosophila* genes encoding homologs of mammalian PSD proteins lead to a reduction in GluRIIA cluster sizes

(A) Confocal images of third instar larval 6/7 NMJs immunolabeled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label postsynaptic glutamate receptor clusters containing the GluRIIA subunit. Inset panels show high resolution terminal boutons. Scale bar = 20 μm. (B) Histogram showing GluRIIA cluster sizes for genotypes represented in A. (C) Quantification of the number of boutons (left) and branches (right) indicative of presynaptic motor neuron morphology.

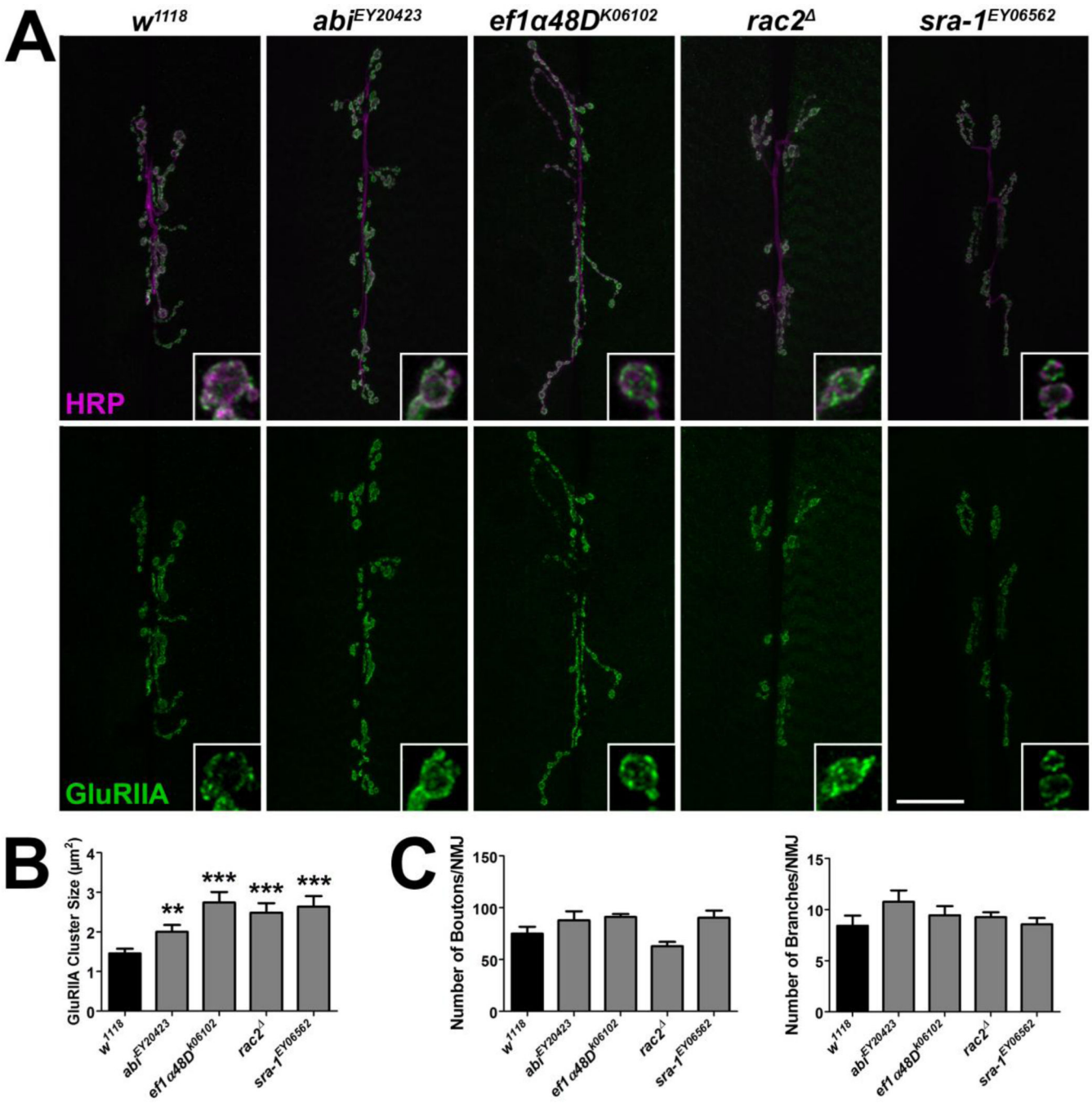

Figure 2. Mutations in *Drosophila* genes encoding homologs of mammalian PSD proteins lead to an increase in GluRIIA cluster sizes

(A) Representative confocal micrographs of 6/7 NMJs immunolabeled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label GluRIIA-containing glutamate receptor clusters. Inset panels show high resolution terminal boutons. Scale bar = 20 μm. (B) Quantification of GluRIIA cluster sizes for genotypes shown in A. (C) Quantification of characteristics representative of presynaptic motor neuron morphology including the number of boutons (left) and branches (right).

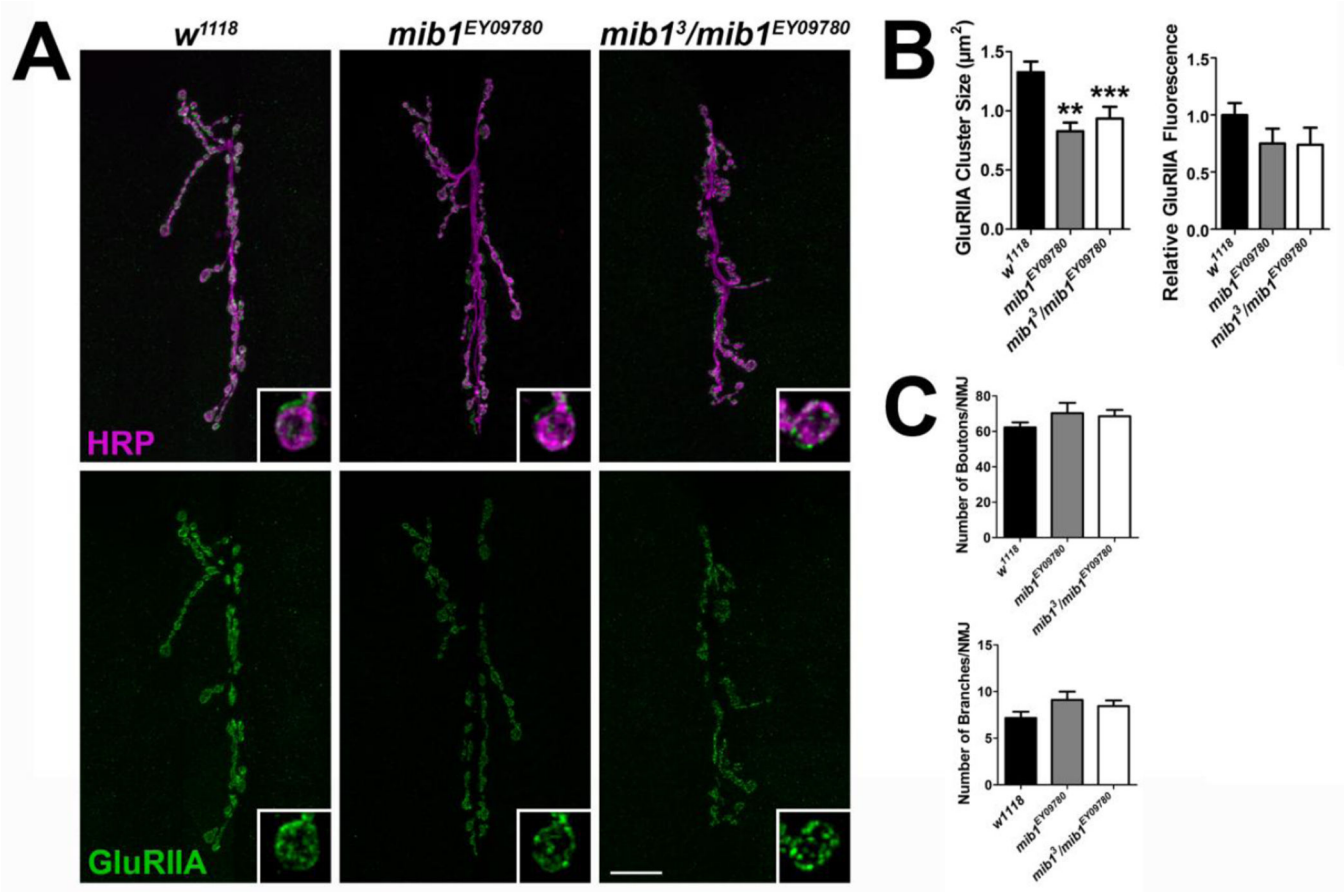

Figure 3. Mib1 is important for the clustering of GluRIIA-containing receptors

(A) Control and *mib1* mutant confocal images showing representative 6/7 NMJs from third instar larvae. Preparations were immunolabled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label GluRIIA-containing glutamate receptor clusters. Inset panels show high resolution terminal boutons. Scale bar = 20 μm. (B) Histograms showing quantification of GluRIIA cluster sizes (left) and GluRIIA relative fluorescence intensity (right) for genotypes shown in A. (C) Quantification of characteristics representative of presynaptic motor neuron morphology including the number of boutons (top) and branches (bottom).

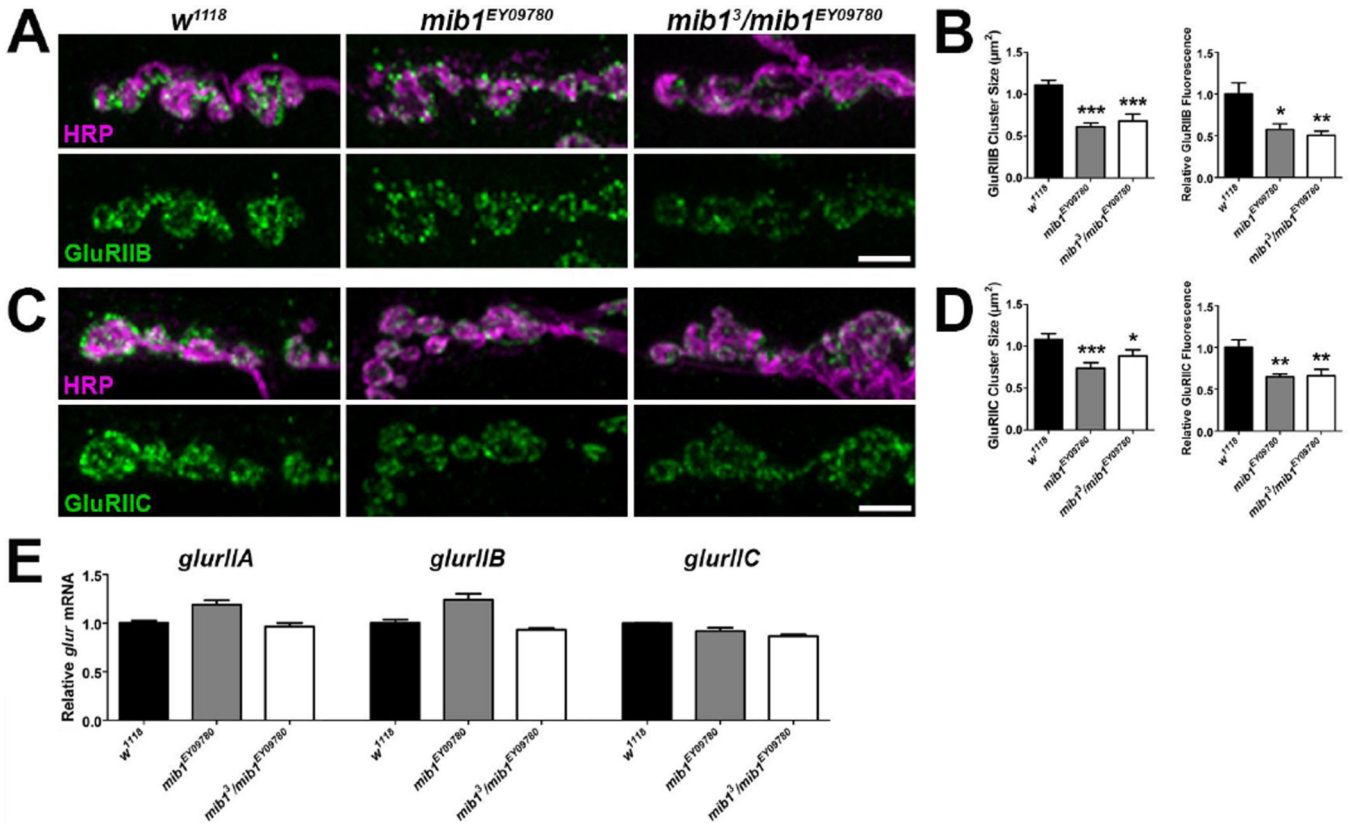

Figure 4. Mib1 positively regulates synaptic levels of GluRIIB and GluRIIC but does not affect *glur* transcript levels

High resolution confocal micrographs showing third instar larvae terminal boutons of the 6/7 NMJ immunolabeled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label GluRIIB-containing glutamate receptor clusters (A) or GluRIIC-containing glutamate receptor clusters (C). Scale bar = 5 μm. (B) Quantification of GluRIIB cluster sizes (left) and relative fluorescence intensities (right) for genotypes shown in A. (D) Quantification of GluRIIC cluster sizes (left) and relative GluRIIC fluorescence intensity (right) for genotypes shown in B. (E) Quantification qRT-PCR ΔΔC(t) values normalized to the control, w^{1118}, using gene-specific primers for g*lurIIA* (left), *glurIIB* (middle), and *glurIIC* (right).

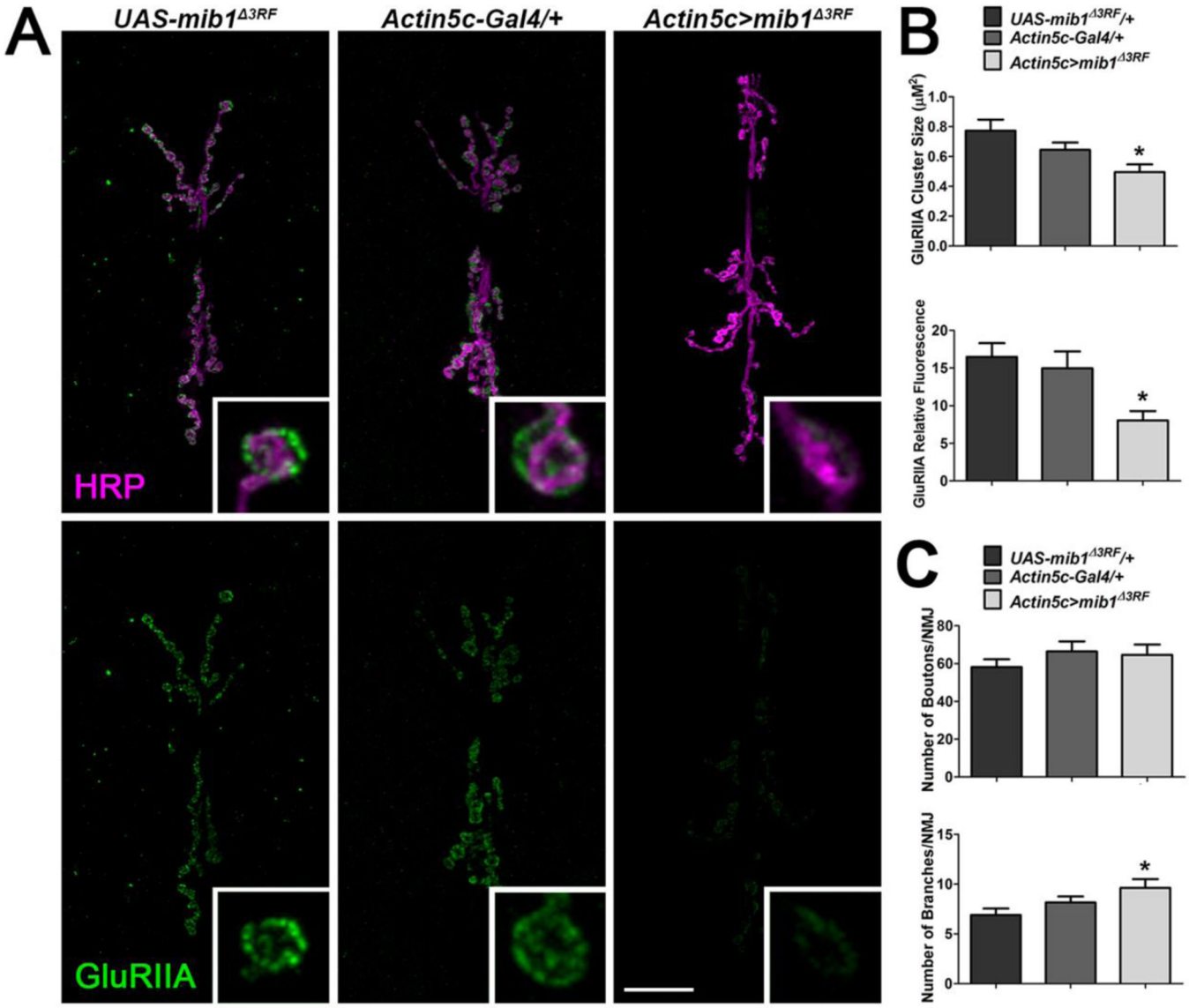

Figure 5. Expression of Mib1 lacking the RING finger domains leads to the loss of synaptic GluRIIA

A *mib1* transgene lacking the region encoding the three C-terminal RING finger domains, *UAS-mib1*$^{\Delta 3RF}$, was expressed in all tissues using the *Actin5c-Gal4* driver. (A) Representative third instar larvae 6/7 NMJs immunolabeled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label postsynaptic glutamate receptor clusters containing the GluRIIA subunit. Inset panels show high resolution terminal boutons. Scale bar = 20 μm. (B) Quantification of GluRIIA cluster sizes (left) and GluRIIA fluorescence intensities (right) for genotypes represented in A. (C) Characterization of presynaptic motor neuron morphology by quantification of the number of boutons (top) and branches (bottom).

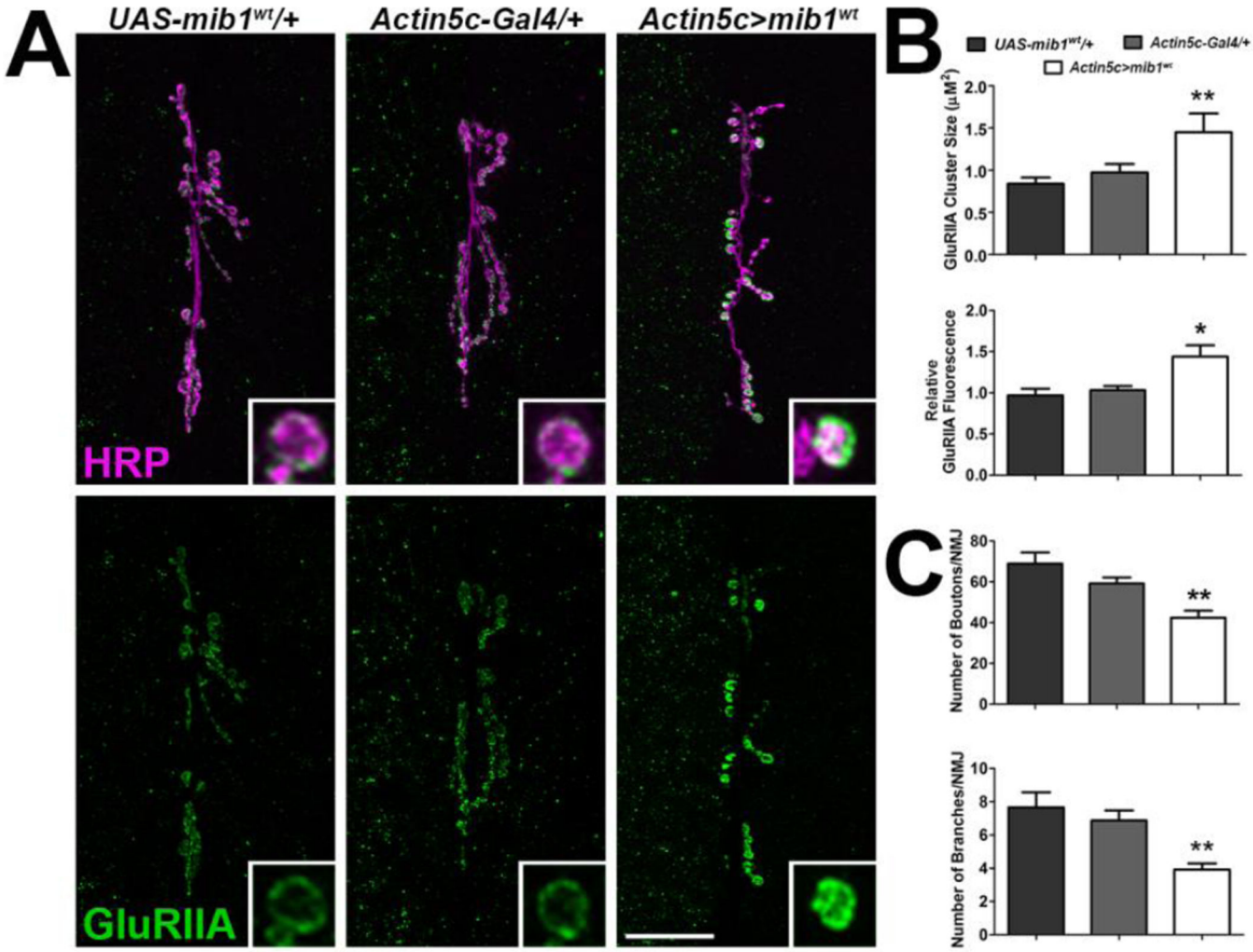

Figure 6. Overexpression of Mib1 leads to an increase in synaptic GluRIIA and a reduction in the size of the presynaptic motor neuron

Overexpression of *mib1* was achieved by expressing *UAS-mib1^{wt}* in all tissues using the *Actin5c-Gal4* driver. (A) Confocal micrographs showing 6/7 NMJs immunolabeled with α-HRP to label presynaptic motor neurons (magenta) and α-GluRIIA (green) to label postsynaptic GluRIIA-containing glutamate receptor clusters. Inset panels show high resolution terminal boutons. Scale bar = 20 μm. (B) Histograms showing quantification of GluRIIA cluster sizes (left) and GluRIIA fluorescence intensities (right) for genotypes represented in A. (C) Histograms showing quantification of the number of boutons (top) and branches (bottom).

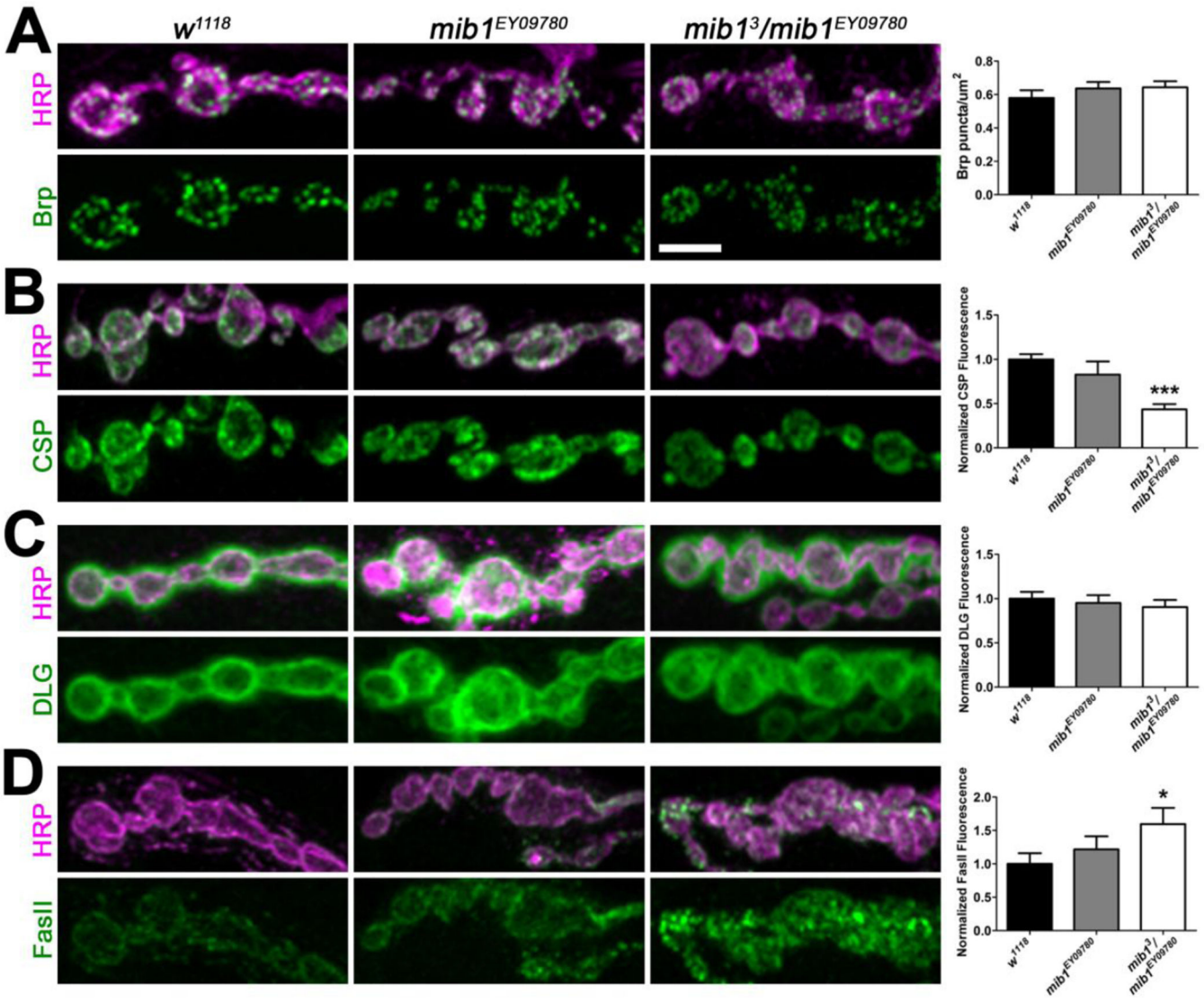

Figure 7. Mib1 is important for the localization of CSP and FasII at the synapse

High resolution confocal micrographs showing terminal boutons of 6/7 NMJs from third instar larvae immunolabeled with α-HRP (magenta) and α-Brp (green, A), α-CSP (green, B), α-DLG (green, C), or α-FasII (green, D). Scale bar = 5 μm. Right histograms show quantification of mean normalized fluorescence intensities.

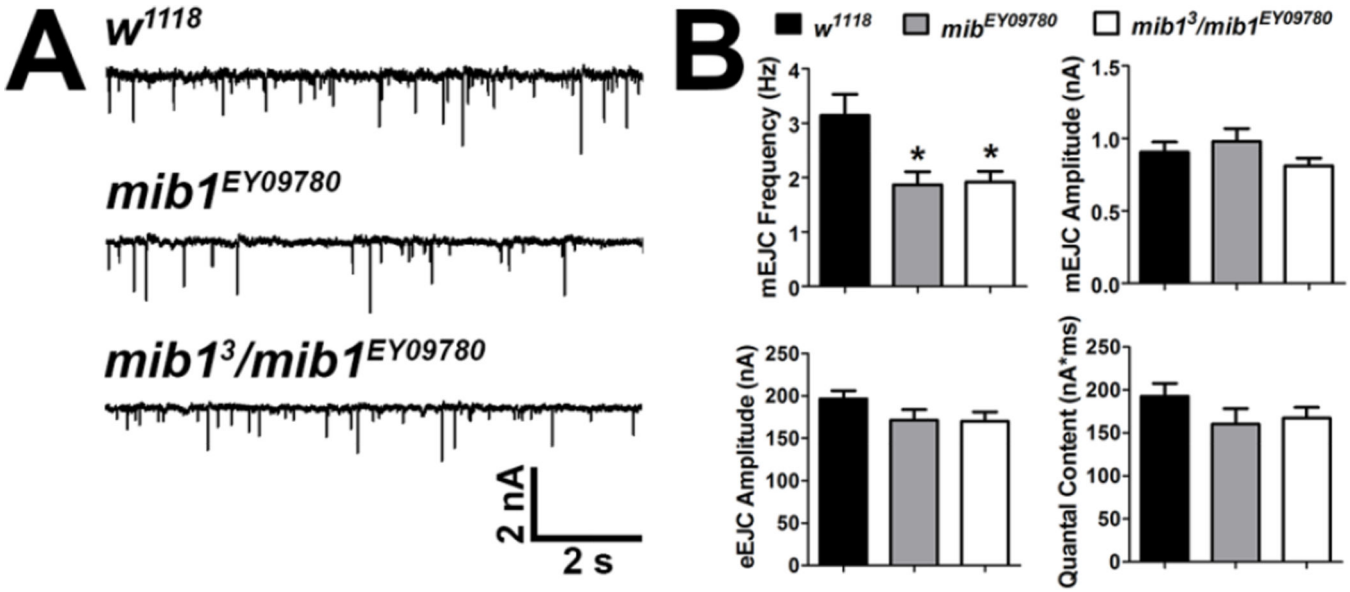

Figure 8. Mib1 negatively regulates spontaneous synaptic transmission

Spontaneous (mEJCs) and evoked junctional currents (eEJCs) were recorded from third instar larvae after voltage clamp of muscle 6 at −60 mV. (A) Representative mEJCs from control and *mib1* mutants. (B) Quantification of mEJC frequency and amplitudes (top) and eEJC amplitudes and quantal content (bottom).

Table 1

Classification of mutations identified in the reverse genetic screen that significantly affected synaptic GluRIIA levels

Function of Gene Product	Percentage
Cell adhesion molecules	8.3%
Cytoskeleton and related	18.8%
GTPases and regulators	25.0%
Kinases and phosphatases	10.4%
Metabolic	4.2%
Other	8.3%
Receptors/channels and transmembrane proteins	6.3%
Scaffold protein	16.7%
Trafficking/motor proteins	2.1%