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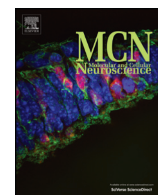

The Notch ligand E3 ligase, Mind Bomb1, regulates glutamate receptor localization in *Drosophila*

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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.

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1. 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 and 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 and Nicoll, 2013; Padamsey and 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 and 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 and 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 and 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

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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 and 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 and 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 and Manuel, 2010; Emes and Grant, 2011). We have previously identified *Drosophila* orthologs for approximately 96% of published mammalian PSD proteins (Liebl and 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).

2. Results

2.1. 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 and 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 and Jan, 1976; Johansen et al., 1989; Ruiz-Canada and 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 screen (Figs. 1A–B, 2A–B), there were no consistent changes observed in the morphology of the presynaptic motor neuron (Figs. 1C, 2C).

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%

2.2. 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 and 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 and Liefke, 2012). To investigate the possibility that Mib1 may influence *GluR* transcript levels by regulating the transcriptional

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