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MODULATION OF HIPPOCAMPAL SYNAPSE MATURATION BY ACTIVITY-REGULATED E3 LIGASE *VIA* NON-CANONICAL PATHWAY

PUSHPA KUMARI, BALAKUMAR SRINIVASAN AND SOURAV BANERJEE *

Synapse Biology Laboratory, National Brain Research Center, NH-8, Nainwal Mode, Manesar 122051, Haryana, India

Abstract—Development of functional synapses is crucial for the transmission and storage of information in the brain. Post establishment of the initial synaptic contact, synapses are stabilized through neuronal activity-induced signals. Emerging studies have implicated ubiquitination; a reversible posttranslational modification, as a key regulatory switch that modulates synapse development through proteasomal degradation. Ubiquitination of proteins is precisely regulated by E3 ligases, a set of enzymes that bind to specific substrates to facilitate the conjugation of monomeric or polymeric ubiquitin. However, the identity of specific E3 ubiquitin ligases that influence activity-dependent maturation of synapses and the mechanism by which ubiquitination of proteins regulate functional synapse development remain elusive. Here, we have identified a RING domain containing E3 ligase, Rnf2, as an activity-regulated factor that modulates glutamatergic synapse development in the hippocampus. Rnf2 is a synapse associated E3 ligase that is stabilized by neuronal activity through self-polyubiquitination. We have shown that neuronal activity shifts the balance toward stabilization of Rnf2 through self-polyubiquitination rather than triggering its degradation through polyubiquitination by Ube3A, an E3 ligase implicated in Angelman Syndrome. Our synapse density measurements and whole-cell patch-clamp recordings have revealed that the loss of Rnf2 function in cultured hippocampal neurons result in the development of 'silent' synapses that lack GluA1 containing functional AMPA receptors. These results provide a plausible mechanistic approach toward understanding how synapse maturation is regulated via the activity-dependent stabilization of Rnf2 through a non-canonical function of polyubiquitination.

Key words: synapse maturation, neuronal activity, polyubiquitination, E3 ligase, AMPA receptor, patch-clamp.

INTRODUCTION

Synapses are specialized microstructures between neurons that are made up of a defined assemblage of pre-synaptic and post-synaptic compartments that work cohesively to execute functions of the neural circuitry. Synapse development is initiated when contact points are established between an axon and a dendrite; following which preassembled packets of proteins along with the synaptic vesicle release machinery are recruited at the presynaptic terminal with a concomitant recruitment of neurotransmitter receptors, signaling molecules, and scaffolding proteins at the postsynaptic compartment (Waites et al., 2005; Tessier and Broadie, 2009). These nascent synapses then undergo activity-dependent maturation to strengthen and maintain a subset of active synapses while the remainder of immature synapses are weakened and then pruned to ultimately sculpt a functional neuronal circuitry (Goda and Davis, 2003; Tessier and Broadie, 2009; Kay et al., 2011). Thus, synapse development is a multi-step process that is spatio-temporally modulated through various regulatory controls including gene transcription in the nucleus, intracellular signaling, protein–protein interaction of ligand-receptor complex as well as various cell adhesion proteins (Waites et al., 2005; Siddiqui and Craig, 2011; West and Greenberg, 2011). Emerging studies have implicated an added layer of regulatory control in the form of ubiquitination of pre- and post- synaptic proteins; a selective post-translational modification that serves as a reversible switch to regulate synapse development (DiAntonio and Hicke, 2004; Mabb and Ehlers, 2010).

Ubiquitination involves the conjugation of the 76 amino acid ubiquitin moiety to a lysine residue of the substrate protein. The reaction is facilitated through three sequential enzymatic reactions involving an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase that work in tandem. Among these, the E3 ubiquitin ligase is a crucial regulator of ubiquitination as it can selectively recruit a subset of target proteins and bind them to the substrate directly (DiAntonio and Hicke, 2004). A growing body of literature has elucidated the role of a few E3 ligases in synapse development (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000; Yi and Ehlers, 2005; Yamada et al., 2013). These studies revealed that E3 ligases are

*Corresponding author. Fax: +91-124-2338910.
E-mail address: sourav@nbrc.ac.in (S. Banerjee).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BCA, bicinchoninic acid; CA, Cornu Ammonis; DG, Dentate Gyrus; DIV, days *in vitro*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, Glial Fibrilary Acidic Protein; GFP, Green Fluorescent Protein; HECT, homologous to the E6-AP carboxyl terminus; MCPG, α-methyl-4-carboxyphenylglycine; MEM, Minimum Essential Medium; mEPSC, miniature Excitatory Post Synaptic Current; NBQX, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*] quinoxaline-7sulfonamide; NMDA, N-methyl D-aspartate; PSD95, Post Synaptic Density 95; RING, Really Interesting New Gene; RNAi, RNA interference; Rnf2, RING finger protein 2; shRNA, short hairpin RNA.

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localized in specific subcellular compartments; such as pre- and post-synaptic sites; to regulate gene expression locally for functional synapse development (Yamada et al., 2013). A growing list of annotated ~617 putative E3 ligases suggest that they are involved in diverse regulatory mechanisms linked with various nervous system functions, particularly the complex stages of synapse development (Li et al., 2008; Mabb and Ehlers, 2010). A prominent example of E3 ligases implicated in synapse development is *highwire* in *Drosophila,* or its ortholog, *rpm-1* in *C. elegans.* Mutation of this E3 ligase leads to aberrant development of the pre-synaptic compartment (Wan et al., 2000; Zhen et al., 2000). Anaphase Promoting Complex (APC), a multi-subunit E3 ubiquitin ligase, has been shown to regulate synapse development in *Drosophila* by regulating the abundance of glutamate receptors at the postsynaptic compartment, thus affecting synaptic transmission (Juo and Kaplan, 2004; van Roessel et al., 2004). Consistent with these observations from invertebrate model organisms, PDZRN3, a RING domain containing E3 ligase has been shown to modulate the surface expression of muscle-specific kinase (MuSK) at the postsynaptic membrane *via* ubiquitination and subsequently regulate neuromuscular junction (NMJ) synapse formation in mice by controlling nicotinic acetylcholine receptor clustering (Lu et al., 2007). The non-redundancy of E3 ligases is further underscored by a recent example, wherein a mutation in the HECT domain containing the E3 ligase, Ube3A, was implicated in Angelman Syndrome which is a neurodevelopmental disorder. Disruption of Ube3A function in the Angelman Syndrome mice model, leads to an increased Arc expression and a concomitant decrease in the number of AMPA receptors at the postsynaptic membrane, resulting in aberrant synaptic transmission (Greer et al., 2010).

Interestingly, the RING domain containing E3 ligase, Rnf2 or Ring1B, a component of Polycomb Repressive Complex 1 (PRC1), has been shown to regulate neuronal differentiation of sub-cerebral projection neurons during the mouse neocortical development (Morimoto-Suzki et al., 2014). Another study has shown that Rnf2 mono-ubiquitinates nucleosomal histone H2A to regulate gene expression both in neural tissues such as cerebellar Purkinje cells and non-neural tissue; such as liver (Zaaroor-Regev et al., 2010). Apart from ubiquitinating histone H2A, Rnf2 has also been implicated in the ubiquitination of various signaling proteins and thereby, is pivotally positioned as a master E3 ligase involved in gene expression control in neuronal as well as non-neuronal systems (Roman-Trufero et al., 2009; Vidal, 2009; Zaaroor-Regev et al., 2010; Dietrich et al., 2012; Morimoto-Suzki et al., 2014). Ube3A and Rnf2 have been shown to mutually regulate each other's function by polyubiquitination in various tissues including brain (Zaaroor-Regev et al., 2010). Ube3A targets Rnf2 for proteasomal degradation *via* the ''canonical" polyubiquitination chain that differs from the multiple-branched ''non-canonical" polyubiquitination chain self-conjugated by Rnf2 for its stabilization (Zaaroor-Regev et al., 2010). This observation suggests that neuronal activity could function as the major control point for Rnf2 activation vs. degradation *via* differential polyubiquitination patterns.

Apart from the polyubiquitination–mediated tagging of synaptic proteins for degradation, a recent study has demonstrated that accumulation of the polyubiquitinated proteins facilitate the assembly of pre-synaptic terminals *via* a non-canonical pathway (Pinto et al., 2016). However, a comprehensive picture of specific E3 ligases that modulate polyubiquitination for non-degradative functions remain elusive. Particularly, the mechanistic details of ubiquitin–mediated control of synapse formation and the role of E3 ligases in different phases of synapse development require further elucidation.

Here, we have identified Rnf2 as a synapse-associated E3 ligase that is regulated by neuronal activity during synapse formation. Our study revealed that neuronal activity has stabilized Rnf2 expression *via* self-polyubiquitination rather than triggering its degradation by Ube3A–mediated polyubiquitination Observations from synapse density measurements and mEPSC recordings have shown that the absence of Rnf2 leads to an increase in the number of immature synapses that are electrically 'silent.' We found that such an increase of silent synapses was concomitant with the impairment of GluA1-subunit containing AMPA receptor insertion into the post-synaptic membrane. These results point toward a novel regulatory mechanism of glutamatergic synapse maturation through activity-dependent control of Rnf2 expression *via* a non-canonical function of polyubiquitination.

EXPERIMENTAL PROCEDURES

Cell culture

Rat and mouse primary hippocampal neuronal cultures were prepared as described previously (Kaech and Banker, 2006). Timed pregnant rats (Sprague Dawley) and mice (C57BL6/J) were generated at the animal house of National Brain Research Center as per methods approved by Institutional Animal Ethics Committee (IAEC). The Ube3A mutant heterozygous mice carrying paternally imprinted Ube3A (Ubiquitin protein ligase 3A) knockout mutation was obtained from The Jackson Laboratory, USA (Stock Number 129-Ube3A^{tm1Alb}/J). These heterozygous mice were mated to obtain homozygous Ube3A null mice at the animal facility of National Brain Research Center. These null mice were further mated to obtain timed pregnant Ube3A null mice for preparation of primary hippocampal neuronal culture. Briefly, hippocampi were dissected from either E18 rat (Sprague Dawley) embryos or E15 mouse (C57BL6/J or Ube3A null mice in C57BL6/J background) embryos. Hippocampi were trypsinized to prepare a single-cell suspension that was plated onto poly-L-lysine (1 mg/ml)-coated coverslips (190–300 cells/mm^2). 300 cells/mm^2 were used for all biochemical experiments, 190 cells/mm^2 were used for electrophysiology and imaging experiments. Neurons were co-cultured with glial cells in Neurobasal Medium (Gibco) containing B27 supplement (Gibco) as indicated.

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