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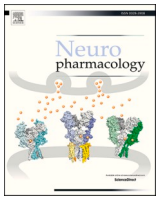

Historical perspective and progress on protein ubiquitination at glutamatergic synapses

Angela M. Mabb [a, b, *]

[a] Neuroscience Institute, Georgia State University, Atlanta, GA, USA
[b] Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA, USA

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ABSTRACT

Transcription-translation coupling leads to the production of proteins that are key for controlling essential neuronal processes that include neuronal development and changes in synaptic strength. Although these events have been a prevailing theme in neuroscience, the regulation of proteins via posttranslational signaling pathways are equally relevant for these neuronal processes. Ubiquitin is one type of posttranslational modification that covalently attaches to its targets/substrates. Ubiquitination of proteins play a key role in multiple signaling pathways, the predominant being removal of its substrates by a large molecular machine called the proteasome. Here, I review 40 years of progress on ubiquitination in the nervous system at glutamatergic synapses focusing on axon pathfinding, synapse formation, presynaptic release, dendritic spine formation, and regulation of post-synaptic glutamate receptors. Finally, I elucidate emerging themes in ubiquitin biology that may challenge our current understanding of ubiquitin signaling in the nervous system.

1. Introduction

Transcription-translation coupling leads to the production of proteins that are key for controlling essential neuronal processes that include neuronal development and changes in synaptic strength (Buffington et al., 2014; Greer and Greenberg, 2008). Although these events have been a prevailing theme in neuroscience, the regulation of proteins via posttranslational signaling pathways are equally relevant for these neuronal processes. Ubiquitin is one type of posttranslational modification (PTM) that covalently attaches to its targets/substrates (Hershko and Ciechanover, 1998). Ubiquitination of proteins play a key role in multiple signaling pathways, the predominant being removal of its substrates by a large molecular machine called the proteasome (Collins and Goldberg, 2017). Ubiquitin can also trigger the degradation of proteins through various structures or modalities such as their removal through endoplasmic-reticulum-associated protein degradation (ERAD), phagocytosis, autophagy through the lysosome and mitophagy through the mitophagosome (Hershko and Ciechanover, 1998).

Free ubiquitin is highly abundant in the nervous system (>20 times compared to muscle) and can be detected at synapses implying that it is a major PTM that regulates essential neuronal processes (Chain et al., 1995; Chapman et al., 1994). Moreover, ubiquitination in the nervous system appears to be specialized, perhaps in an effort to accommodate the metabolic demands of postmitotic neurons over a lifetime. For example, ubiquitin machinery in postmitotic neurons includes brain-specific ubiquitin components (Berti et al., 2002; Tai et al., 2010; Wilkinson et al., 1989) and recently discovered extracellular exposed membrane proteasomes (Ramachandran and Margolis, 2017). These membrane structures are thought to degrade ribosome-associated nascent polypeptides such as immediate early genes (Ramachandran et al., 2018; Turker et al., 2021). Although ubiquitin was found to be expressed in the mammalian brain in 1978 (Scherrer et al., 1978), prior to the 1990s, there was a dearth of research describing ubiquitin signaling in the nervous system (57 articles in total). However, in the past 30 years, there has been a massive expansion of literature describing ubiquitin functions in the brain (over 8000 articles in total). This increase has most likely been driven by an accumulation of seminal studies describing disordered protein ubiquitination and causative mutations of ubiquitin enzyme genes in numerous neurological conditions (Cheon et al., 2019; George et al., 2018; Lim et al., 2020; Zajicek and Yao, 2020; Zheng et al., 2016).

In this review, I discuss major functions of protein ubiquitination in the nervous system that occur at glutamatergic synapses with a particular emphasis on axon pathfinding, synapse formation, presynaptic

* Georgia State University 100 Piedmont Ave SE Atlanta, GA 30303 USA.
E-mail address: amabb@gsu.edu.

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release and regulation of postsynaptic glutamate receptors. Finally, I elucidate emerging themes in ubiquitin biology that may challenge our current understanding of ubiquitin signaling in the nervous system.

2. The ubiquitin cascade: as easy as E1, E2, E3

Protein ubiquitination is an ATP-driven event that relies on an enzymatic cascade consisting of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and in some cases an E4 ligase (E4). In this series, ATP hydrolysis facilitates the attachment of a pre-primed ubiquitin to an active cysteine residue of the E1 creating a thioester intermediate. The ubiquitin is then transferred from its E1-loaded complex to an active site cysteine residue of the E2. The ubiquitin-loaded E2 then engages with an E3 to position the ubiquitin for transfer to its substrate (Pickart, 2001). In certain cases, an E4 can coordinate with an E3 to facilitate the elongation of ubiquitin chains to promote efficient degradation by the ubiquitin proteasome system (UPS) (Koegl et al., 1999). There are 1–2 E1, 30–50 E2, and 600–700 E3 genes (Zheng and Shabek, 2017). The way in which ubiquitin is transferred to a substrate is dependent on the type of E3 enzyme catalytic domain and it is generally accepted that the E3 is the predominant determinant for substrate selectivity.

Canonically, ubiquitin is covalently conjugated to a lysine residue of the substrate, however other amino acid residues such as cysteine, serine, and threonine are known to be targeted for ubiquitination (Cadwell and Coscoy, 2005; Mabbitt et al., 2020; McClellan et al., 2019; Pao et al., 2018). Ubiquitin can also be assembled on a substrate in multiple configurations creating a complex code, either through a single attachment (often referred to as mono-ubiquitination) or as a series of polymeric chains (often referred to as polyubiquitination) that can assemble as M1-, K6-, K11-, K27-, K29-, K33-, K48- and K63-linkages or as a series of heterotypic or branched structures (Yau and Rape, 2016). These distinct assemblies lead to different signaling events that broadly participate in biological functions (Fig. 1).

As ubiquitin is assembled via a series of enzymes, it can be readily reversed by the presence of deubiquitylases (DUBs). These diverse group of enzymes (80–100 genes representing 7 families) are specialized to remove select types of ubiquitin moieties (Clague et al., 2019; Komander et al., 2009).

3. History of protein ubiquitination in the nervous system

In 2004, Aaron Ciechanover, Avram Hershko, and Irwin Rose were awarded the Nobel Prize in Chemistry for their discovery of ubiquitin-mediated protein degradation, demonstrating the importance of this pathway in key biological functions (Goldberg, 2005). Protein degradation has also been a major pathway regulating all aspects of nervous system function. One of the first descriptions for the role of protein ubiquitination in synaptic function of the nervous system was characterized in Drosophila. A genetic screen was conducted to identify genes that were critical for giant fiber (GF) command neurons in the thoracic muscles to regulate the escape jump response. In this screen, the X-linked gene bendless was identified as a factor that eliminated the jump response and disrupted synaptic transmission between the GF neuron and the tergotrochanter motor neuron innervating the thoracic muscle (Thomas and Wyman, 1984). Bendless was later identified as an E2 that was highly expressed in the Drosophila nervous system during development with homology to human Ubc13 (Muralidhar and Thomas, 1993; Oh et al., 1994; Yamaguchi et al., 1996). In humans, Ubc13 is more highly expressed in the muscle and testis (Yamaguchi et al., 1996). This E2 was later identified to selectively assemble K63-linked ubiquitin chains, which are critical for providing scaffolds for cellular signaling pathways (Fig. 1) (Bai et al., 2018; Hofmann and Pickart, 1999). These findings established a requirement for nondegradative protein ubiquitination pathways in mediating synaptic development.

The recognition of protein proteolysis as a means to regulate long-term changes in synaptic strength at glutamatergic synapses started to emerge around the 1980's where it was proposed that the proteinase calpain could degrade a plasma membrane anchored cytoskeleton bound protein called fodrin as a means to alter the abundance of glutamate receptors (Baudry et al., 1983; Lynch and Baudry, 1984; Siman et al., 1984). It was not until the late 1980's where the link between the removal of proteins and learning-related behaviors was established. Serotonin (5-HT)-mediated long-term facilitation (LTF) of

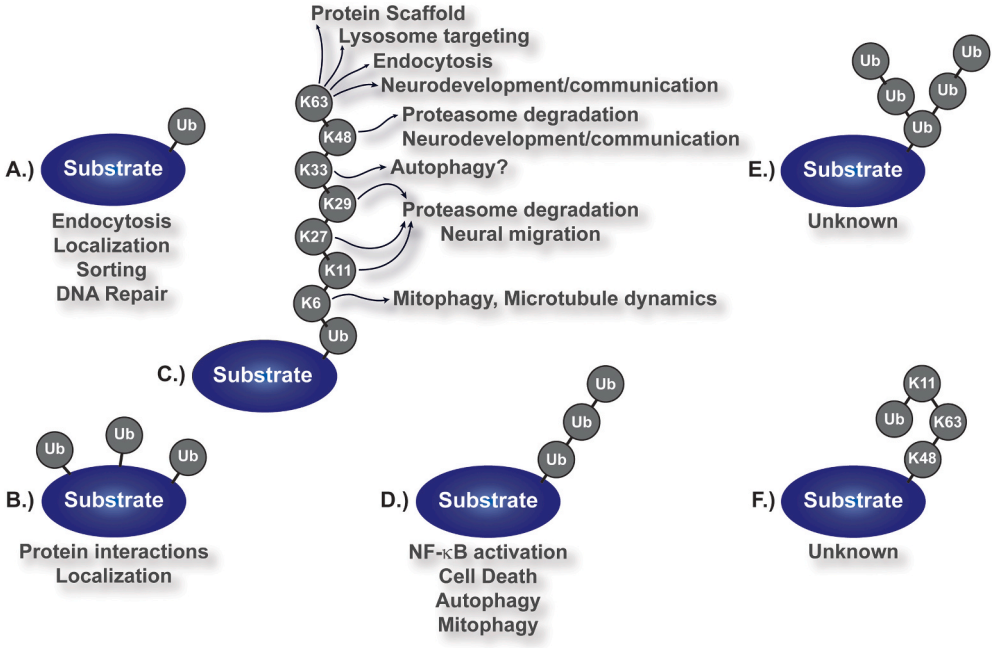

Fig. 1. Function of ubiquitin linkage types in the nervous system. Schematic of the different ubiquitin linkage configurations and their functions in the nervous system. A.) Monoubiquitination, B.) Multi-monoubiquitination, C.) Polyubiquitination with assembly via K6-, K11-, K27-, K29-, K33-, K48-, or K63-homotypic linkages. D.) Linear ubiquitination, E.) Branched heterotypic ubiquitin linkages, F.) Mixed heterotypic ubiquitin linkages.

the glutamatergic sensorimotor neuron synapse in Aplysia increases synaptic strength underlying long-term sensitization (LTS) of the gill- and siphon-withdrawal reflex (Frost et al., 1985). This form of plasticity and defensive behavior requires transcription and protein synthesis (Kandel, 2001). However, LTS was also found to be associated with a posttranslational chronic decrease of isoforms of the regulatory (R) subunit of the protein kinase A (PKA) complex with no effect on the expression of PKA catalytic subunits. Thus, the loss of the R subunit led to an increase of PKA activity in the cell (Bergold et al., 1992; Greenberg et al., 1987). The mechanism explaining the reduction of the R subunit was due to the formation of high molecular weight R conjugates, which were identified as being ubiquitin positive (Chain et al., 1995; Hegde et al., 1993). Subsequent studies provided strong evidence that R subunit ubiquitination was the signal that led to its removal by the UPS allowing the transition to long-term changes in synaptic strength (Chain et al., 1995, 1999; Hegde et al., 1993). Later, prolonged PKA activity was found to be critical for maintaining the late phase of long-term potentiation (LTP) at mammalian synapses (Huang and Kandel, 1994). Although R subunit ubiquitination and UPS-dependent removal has yet to be measured at mammalian glutamatergic synapses in response to LTP, Praja2 was identified as an E3 (Yu et al., 2002) that could potentially degrade R subunits in mice. Knockdown of Praja2 in the hippocampus by siRNA blunted LTP induced at hippocampal perforant path glutamatergic synapses (Lignitto et al., 2011).

The ubiquitin C-terminal hydrolase (Ap-uch) is a gene that associates with the proteasome which increases its proteolytic activity (Eytan et al., 1993; Hadari et al., 1992). This gene was found to be rapidly upregulated in Aplysia sensory neurons in response to long-term facilitation by serotonin (5-HT) (Hegde et al., 1997). Injection of an anti-Ap-Uch antibody or antisense oligonucleotide for Ap-uch was able to block 5-HT induced LTF but had no effect on short-term facilitation (Hegde et al., 1997). Cumulatively, these studies established a role for protein ubiquitination pathways as key regulators of LTF and the formation of long-term memories. Similar rulesets were later established at glutamatergic synapses in mammals (reviewed in (Mabb and Ehlers, 2010)). Moreover, long-term decreases in synaptic strength known as synaptic depression also require ubiquitin pathway constituents (Citri et al., 2009; Colledge et al., 2003; Fioravante et al., 2008; Hou et al., 2006) thus solidifying the requirement of ubiquitination in mediating long-term changes in synaptic strength at glutamatergic synapses.

4. Ubiquitin-dependent structural maintenance of proteins at glutamatergic synapses

Early studies found that ubiquitin conjugates could be detected in isolated synaptic membrane and postsynaptic density (PSD) fractions from rat forebrain (Chapman et al., 1994). Modulation of neuronal activity at glutamatergic synapses with the Na^+ channel blocker tetrodotoxin (TTX) and inhibition of gamma-aminobutyric acid ($GABA_A$) receptors with bicuculline was found to modulate proteasome activity, dramatically altering the composition of postsynaptic density (PSD) proteins in a bidirectional manner. Some of these protein “ensembles” (e.g. Shank, GKAP, and AKAP75/150) were identified as being directly ubiquitinated leading to the hypothesis that the ubiquitination and subsequent degradation of key PSD proteins by the proteasome are required for synaptic remodeling in response to neuronal activity (Djakovic et al., 2009; Ehlers, 2003). Synaptic protein degradation upon global changes in network activity was further supported by a recent isotope labeling study in cortical neurons, where protein half-lives were stabilized following a decrease in neuronal activity with TTX (Heo et al., 2018). However, unlike the previous studies, there was a subtle but insignificant increase in protein turnover rates with bicuculline treatment, which was suggested to be due to high basal excitatory activity that occurs when culturing primary neurons. Upon metabolic labeling of endogenous proteins in mice, synaptic proteins were found to have longer half-lives compared to the cytosolic pool indicating their assembly into stable complexes. However, protein half-lives at synapses could be reduced when animals experienced environmental enrichment, which is known to elicit structural changes at excitatory synapses important for learning (Heo et al., 2018).

The 20S proteasome core particle complex, which is the proteolytic component of the 26S proteasome could be detected in about 60% of synapses in hippocampal neuron cultures (Patrick et al., 2003). It was later found that increased synaptic activity via KCl-induced membrane depolarization caused 20S proteasome-associated proteins to be retained in dendritic spines (Bingol and Schuman, 2006). Treatment of neurons with KCl or N-methyl-D-aspartate (NMDA) increased the activity of the proteasome. KCl-induced proteasome activity was found to depend on NMDA as demonstrated by blocking its translocation with the NMDA antagonist, AP5. Increasing excitatory activity via bicuculline also caused the translocation of the 20S proteasome into dendrites. This localization change was later found to be dependent on the transcriptional repressor and actin binding protein Nucleus accumbens-associated 1 (NAC1) (Shen et al., 2007), which may functionally anchor the proteasome to the actin cytoskeleton in dendritic spines (Bingol and Schuman, 2006). The recruitment of 20S proteasomes to spines also required CaMKIIα, which could directly interact with key 20S proteins, serving as a scaffold for activity-dependent recruitment of the 20S into dendritic spines. Knockdown of CaMKIIα using RNAi reduced the recruitment of the 20S associated protein, Rpt1 to dendritic spines upon NMDA stimulation. This translocation deficit could be over rescued by expression of a RNAi-resistant version of CaMKIIα. CaMKIIα translocation was also found to be important for the degradation of K48-linked ubiquitin chains in dendritic spines as expression of a CaMKIIα mutant that could not translocate 20S led to a buildup of these chains selectively in dendritic spines (Bingol et al., 2010). Cumulatively, these findings provided strong evidence that neuronal activity leads to ubiquitination and subsequent removal of proteins at excitatory synapses. However, the enzymatic machinery that was required to promote ubiquitination of synaptic targets had yet to be resolved.

5. Ubiquitin-dependent control of presynaptic processes at glutamatergic synapses

While the majority of studies related to protein ubiquitination have focused on postsynaptic mechanisms, there are a series of studies that have elucidated the role of protein ubiquitination on presynaptic processes that are critical for formation of glutamatergic synapses across species. Since the identification of the E2 bendless as a critical mediator of synapse formation (Muralidhar and Thomas, 1993; Oh et al., 1994; Thomas and Wyman, 1984), there have been numerous studies linking protein ubiquitination that include axon growth and guidance, presynaptic bouton formation, and neurotransmitter release (DiAntonio and Hicke, 2004; Grill et al., 2016; Hamilton and Zito, 2013; Huang and Bonni, 2016; Tian and Wu, 2013). On a gross scale, increases in protein ubiquitination and proteasome activity coincide with the peak of synaptogenesis (Chen et al., 2011; Franco et al., 2011; Keller et al., 2000; Petersen et al., 2010) and proteasome subunits and ubiquitin conjugates can be found in presynaptic terminals (Chain et al., 1995; Speese et al., 2003). Ubiquitin, E1 and components of the 20S proteasome were also identified in retinal growth cones and proteasome activity was critical for chemotropic turning mediated by Netrin-1 (Campbell and Holt, 2001). Moreover, selective inhibition of the proteasome in axons increases the density of vesicular glutamate transporter 1 (VGluT1) positive presynaptic terminals (Pinto et al., 2016a) that occurs on a similar timescale to synapse formation (Friedman et al., 2000) and requires the formation of K48- and K11-linked ubiquitin chains (Pinto et al., 2016a, 2016b). Collectively, these findings provided strong evidence for a major role of protein ubiquitination in axon function and synapse formation.

5.1. Axon growth and guidance

In Aplysia glutamatergic sensory neurons, inhibition of the proteasome in the presynaptic terminal increases neurite length and branching and increases synapse number between postsynaptic motor neurons (Zhao et al., 2003). In *C. elegans*, the E3 ligase PHR was one of the first factors to control axon growth (discussed below (Schaefer et al., 2000)) and the *C. elegans* homolog for the human SCF E3 F-box protein $SCF^{\beta-TrCP}$, LIN-23 modulated axon outgrowth in glutamatergic mechanosensory neurons (Mehta et al., 2004). In the Drosophila CNS, the HECT E3 ligase Nedd4 (DNedd4) was found to be essential for midline crossing of CNS axons. DNedd4 interacts with Commissureless (Comm), a membrane protein whose function is critical for axons to cross the midline via regulation of Robo expression (Myat et al., 2002). A loss of DNedd4 in Drosophila S2 cells resulted in an increase in surface levels of Comm, suggesting that it controls the trafficking of this receptor. DNedd4 depletion in vivo led to a thickening of axon tracts that should have formed commissures and altered midline crossings, which is similar to a loss of Comm (Georgiou and Tear, 2002). Overexpression of Comm led to multiple axon crossings along the midline that reduced Robo expression. This phenotype could be enhanced upon overexpression of DNedd4 but was dampened upon expression of a DNedd4 that lacked its HECT domain E3 catalytic activity (Myat et al., 2002). These findings suggested that DNedd4 regulated the ubiquitination of Comm, which was critical for its ability to regulate Robo expression for proper axon guidance. However, another study challenged this finding showing that expression of a Comm mutant that cannot be ubiquitinated by DNedd4 was still able to rescue axon guidance deficits. Furthermore, the axon guidance deficits upon modulation of DNedd4 was not able to be reproduced (Keleman et al., 2005). In mammals, it was suggested that Nedd4 interacting proteins (Ndfip-1/2), which are adapters that regulate Nedd4 were analogous to Drosophila Comm. Overexpression of Nfip-1 or -2 decreased surface Robo and increased its ubiquitination in cultured nonneuronal cells that could be blocked with the HECT E3 ligase inhibitor, Heclin (Gorla et al., 2019). Cumulatively, these findings suggest a role for protein ubiquitination in regulating axon guidance.

5.1.1. PHR E3 ligase

One of the most heavily studied ubiquitin signaling pathways in presynaptic development are related to PAM/Highwire/RPM-1 (PHR) proteins. These large sized proteins (>400 kDa) are RING E3 ligases that are critical for axon guidance and degeneration (Grill et al., 2016). PHR proteins are conserved across species that include Regulator of Presynaptic Morphology (RPM-1) (*C. elegans*), Esrom (*D. rerio*), Highwire (*D. melanogaster*), PAM, Highwire, and RPM-1 (Phr1) (rodents), and PAM/MYCBP2 (*H. sapiens*). For brevity, I refer to these as PHR. PHR proteins were first identified in regulating synapse formation in *C. elegans*. *C. elegans* mechanosensory neuron axons express VGLUT indicating that they release glutamate, and include PLM and ALM mechanosensory neurons that pass through the nerve ring to line the pharynx (Serrano-Saiz et al., 2013). A fluorescence-based screen to identify genes that alter presynaptic vesicle localization around the nerve ring identified rpm-1 as a positive regulator of vesicle assembly (Schaefer et al., 2000). PHR was found to be expressed in the nerve ring and ventral cord neuropil. Mutant phr animals lacked patches around the ventral nerve cord and were missing puncta that lined the nerve ring neuropil. Moreover, phr mutants had defects in synaptic branching and ectopic growth of PLM axons. These deficits in presynaptic patterning could be rescued upon presynaptic expression of PHR (Schaefer et al., 2000). FSN-1 is an F-box protein that assembles into a multisubunit Skp1-Cul1-F-box protein (SCF) E3 ligase complex (Deshaies and Joazeiro, 2009). PHR was found to form a complex with FSN-1, CUL-1, and SKR-1 (Liao et al., 2004). Notably, fsn-1 mutant PLM axons extend beyond the ALM cell body but can be rescued upon deletion of the MAPKKK dlk-1 suggesting a role for FSN-1-dependent ubiquitination of DLK-1 (Baker et al., 2015).

A genetic screen for regulators of the glutamatergic neuromuscular junction (NMJ) in Drosophila third instar larvae identified highwire/phr, which exhibited exuberant growth of presynaptic NMJ terminals and an increase in the abundance of presynaptic boutons (Wan et al., 2000). Phr was found to be expressed in periactive zones of the presynaptic terminal. Although phr mutant flies had larger presynaptic terminals, they had a surprising decrease in synaptic transmission that included a reduction in quantal release. Using another genetic screen for alterations in NMJ formation in overexpressing lines, the DUB fat facets was identified as a factor that increased presynaptic size and decreased synaptic transmission in a similar manner to the loss of phr (DiAntonio et al., 2001). Combined loss of function of phr and fat facets could suppress presynaptic deficits; however, this manipulation did not suppress the presynaptic overgrowth phenotype. Combined, these studies indicated that a balance of protein ubiquitination and de-ubiquitination are critical for proper presynaptic formation and further suggested that Phr and Fat facets exert their actions on similar substrates with other DUBs that work on additional Phr substrates.

Another potential substrate for Phr was identified in a yeast two-hybrid screen against interactors for the tumor suppressor, Tuberin (TSC2) (Murthy et al., 2004). Drosophila dTsc1 and dTsc2 co-expression in flies leads to a decrease in eye size. Removal of phr in co-expressing male flies enhanced the small eye phenotype suggesting a genetic interaction between highwire and dTsc1/2. In rat cortical neurons, TSC2 was found to interact with Phr1. In mammals, Phr1 could increase TSC2 ubiquitination that required its E3 ligase activity (Han et al., 2008).

Aspects of PHR proteins were also found to be conserved in vertebrates. A relationship between PHR proteins and Tuberin was identified in retinal axons in zebrafish. Disruption of the PHR orthologue, esrom was found to alter the bundling, target selection and topographic mapping of retinal axons innervating the tectum (D'Souza et al., 2005). Another role for PHR was established in the development of the habenular commissure. This commissure was absent in PHR mutants, which included mutants that lacked the C-terminal catalytic RING and Tuberin binding domains. These findings demonstrated that PHR proteins are essential for midline crossing in the dorsal diencephalon and suggested that PHR suppression of mTOR signaling is a conserved mechanism that controls axon pathfinding (Hendricks et al., 2008).

PHR is also expressed in the CNS and PNS in mice (Burgess et al., 2004). Reducing PHR in rat cortical neuron cultures led to an increase in TSC2 and altered mTOR signaling (Han et al., 2008). Deletion of Phr1 resulted in embryonic lethality due to defects in axonal innervation of the diaphragm (Bloom et al., 2007; Burgess et al., 2004). Notably, Phr1 constitutive KO mice lacked retinal ganglion axon innervation within the lateral geniculate nucleus of the thalamus, lacked the anterior commissure, had corpus callosum narrowing, loss of corticofugal and thalamocortical projections in the subcortical telencephalon, and a shortened habenulopenducular tract. To determine if these effects were acting on DLK ubiquitination, Phr1 mice were crossed with DLK KO mice. However, a loss of DLK did not suppress axon phenotypes and there was no evidence of increased DLK protein in Phr1 KO mice. In another study, an embryo mouse mutant called Magellan had altered motor neuron pathfinding and was mapped to the Phr1 gene. In this model, dorsal root ganglion (DRG) sensory neurons were also shown to be affected (Lewcock et al., 2007), where axons in the upper thoracic and lower cervical segments were inappropriately targeted to the DRG and there was axon overgrowth that included abnormal growth cone morphology and dynamics in cultured sensory axons. Phr1 was found to localize with microtubules, whereas its signaling partner DLK was associated in F-actin enriched growth cones. Inhibition of p38MAPK, a downstream effector of DLK or taxol, the microtubule stabilizer, decreased microtubule disorganization in the Phr mutants. A microtubule phenotype was also identified in cultured forebrain neurons from phr mutant zebrafish. Contrary to the previous study, inhibition of p38MAPK did not rescue the microtubule phenotype. Moreover, low dose treatment with taxol phenocopied the microtubule disorganization

in Phr mutants instead of rescuing deficits (Hendricks and Jesuthasan, 2009). Interestingly, treatment of neurons with the microtubule depolymerizing agent nocodazole was able to suppress microtubule defects and rescue formation of the habenular commissure in vivo providing additional evidence of Phr's role in mediating microtubule disassembly.

A homolog of *C. elegans* FSN-1, Fbxo45 was found to interact with Phr in a proteomics screen. Knockout of Fbxo45 in mice had similar phenotypes to Phr KO mice that included alterations in the development of axon fiber tracts such as loss of the anterior commissure and reduced corticofugal and thalamocortical projections (Saiga et al., 2009). In addition, neural migration from the intermediate zone was impaired in Fbxo45 KO embryos. Upon evaluation of the known downstream effectors of Phr1, there was no difference in the expression of ALK, DLK and TSC2 or the phosphorylation of ERK, p38MAPK, or JNK suggesting an unidentified pathway or set of proteins were being regulated by Fbxo45. This was resolved in part by the identification of mammalian uncoordinated-13 (Munc-13) as a potential substrate (described in section 5.2.2) (Tada et al., 2010) and nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), which were found to be important for Phr effects on axon stability and degeneration in mice and flies (Babetto et al., 2013; Desbois et al., 2018; Xiong et al., 2012; Yamagishi and Tessier-Lavigne, 2016). Although Munc-13 is not involved in neural migration or axon guidance, NMNAT2 has been suggested to contribute to axon growth in mice making this an attractive candidate (Gilley et al., 2013; Hicks et al., 2012). Remarkably, PHR was recently found to have esterification activity which allows for noncanonical ubiquitination of targets on non-lysine residues with a bias for threonine. This type of ubiquitination was found to occur on NMNAT2 suggesting that atypical ubiquitination could be a main factor in PHR-dependent regulation of its presynaptic functions (Pao et al., 2018). Cumulatively, these findings demonstrate a major role for the PHR E3 ligase in axon guidance and microtubule organization across species; however, there appears to be some degree of divergence in downstream effector pathways between species and neuron types.

5.1.2. APC E3 ligase complex

Although the E3 ligase complex anaphase-promoting complex (APC) is traditionally associated with cell cycle control, APC subunits were found to be expressed in postmitotic terminally differentiated neurons. Purification of APC from brain suggested that this complex was active as it supported ubiquitination of known substrates suggesting that it may regulate the ubiquitination of a unique set of substrates in postmitotic cells (Gieffers et al., 1999). The majority of studies on APC have been conducted in cerebellar granule cells, which are glutamatergic neurons that project to Purkinje cell neurons in the cerebellar cortex. As stated previously, APC was found to be enriched in the nucleus of cerebellar granule cell postmitotic neurons that included one of the regulatory proteins for APC, Cdh1 (Cadherin 1). RNAi-mediated depletion of Cdh1 in postnatal developing cerebellar granule neurons led to an increase in axonal length with no significant effects on axon branching. Furthermore, overexpression of a dominant negative catalytic inactive mutant of APC or an inhibitor of APC, Emi1 also increased axon length (Konishi et al., 2004). The ability of APC^{Cdh1} to control axon growth also requires its nuclear localization (Stegmuller et al., 2006). Interestingly, the transcriptional repressor, ski-related novel protein N (SnoN) was identified as a substrate for APC^{Cdh1}. RNAi-mediated knockdown of SnoN dramatically reduced axon length in cerebellar granule and cortical neurons whereas overexpression of a SnoN APC^{Cdh1} recognition motif mutant increased axon length. Further studies supported a role for Smad2 in regulating SnoN degradation by APC^{Cdh1} (Stegmuller et al., 2008).

A repressor of basic helix-loop-helix (bHLH) transcription factors, DNA binding 2/inhibitor of differentiation 2 (Id2) is a short-lived protein that was identified as another potential substrate for APC (Lasorella et al., 2006). APC^{Cdh1} was shown to modulate Id2 half-life and promote its ubiquitination in vitro in a manner that was dependent on its APC recognition motif. RNAi-mediated knockdown of Cdh1 in cerebellar granule neurons increased the levels of Id2. Expression of Id2 that lacked the APC recognition motif increased axon length, which phenocopied the loss of Cdh1. Interestingly, APC^{Cdh1} was shown to target another E3 ligase Smurf1 for degradation, functioning to activate the downstream effector GTPase, RhoA (RhoA is a substrate for Smurf1) (Kannan et al., 2012b). The RhoGAP p250GAP was another factor that was found to interact with APC^{Cdh1} and was suggested to be ubiquitinated but not degraded by the proteasome (Kannan et al., 2012a). Cumulatively, these findings led to a model where APC-mediated degradation of Id2 serves as a switch for the engagement of bHLH target genes such as the Nogo receptor to inhibit axon growth. In parallel, APC also functions to inhibit axon growth by targeting Smurf1 for degradation and p250GAP for ubiquitination to control RhoA activity. The concerted actions of this pathway inhibit axon growth. How APC activity is turned on to target these substrates to terminate axon growth has yet to be established.

5.1.3. TRIM E3 ligase

Developmental axon branching is an important process that allows neurons to innervate multiple targets. Not surprisingly, protein ubiquitination has been shown to regulate axon branching in combination with the cellular machinery that promotes membrane growth and extension. In *C. elegans*, disruption of the tripartite motif (TRIM) RING E3 ligase gene, madd-2 disrupted axon branching and guidance in ventral glutamatergic ADL, PLM, and AVM sensory neurons but not DA and DB cholinergic motor neurons (Hao et al., 2010). Moreover, overexpression of MADD-2 resulted in increased axon outgrowth and ectopic branching of ALM neurons that could be suppressed by disrupting UNC-40 (also known as DCC, the receptor that binds Netrin). In evaluating AVM neurons, axon deficits in madd-2 in combination with unc-6 (Netrin) or unc-40 mutants were not additive suggesting these factors act in the same pathway. In Drosophila, similar findings with the TRIM E3 ligase gene asap were found in class IV sensory axons (Morikawa et al., 2011).

In vertebrates, the conserved TRIM E3, TRIM9 was expressed at axon filopodia tips and was also found to interact and localize with the axon guidance protein, Deleted in Colorectal Cancer (DCC) in axon shafts (Winkle et al., 2014). Deletion of TRIM9 in mice led to aberrant axon branching and thickening of the corpus callosum. Overexpression of TRIM9 enhanced Netrin-dependent stimulation of axon branching in cortical neurons that required its N-terminal RING domain, whereas deletion of TRIM9 increased baseline axon branching in the absence of Netrin treatment. TRIM9 deletion increased the formation of SNARE complexes (proteins that are crucial for mediating exocytosis) and vesicle exocytosis. Notably, TRIM9 interacted with the vesicle fusion protein, SNAP25 and treatment with Netrin-1 decreased this interaction. Expression of a TRIM9 mutant that could not bind to SNAP25 was unable to suppress axon branching in TRIM9 KO neurons. A mechanistic understanding of the interplay between TRIM9 and its unknown substrates was in part provided by Plooster et al. (2017) where TRIM9 deletion decreased netrin-dependent clustering and multimerization of DCC, an important event for axon outgrowth and attraction. Cortical extracts from TRIM9 KO mice also had increased phosphorylation of the DCC downstream effector FAK. TRIM9 was shown to modulate DCC ubiquitination; however, this was not a type of ubiquitination that led to proteasome-dependent degradation. Notably, mutation of putative DCC ubiquitination sites increased the growth cone enriched focal adhesion kinase (FAK) phosphorylation and enhanced DCC interaction with FAK. Taken together, these findings provided strong evidence that TRIM9-mediated interactions with DCC and SNAP25 inhibit exocytosis and serve as a brake for axon branching. Upon engagement with Netrin-1, TRIM9 dissociation from DCC triggers its multimerization to induce phosphorylation of its downstream signaling factor FAK, facilitating SNARE-dependent exocytosis to increase axon branching.

Interestingly, in a follow-up study, deletion of TRIM9 was also found to increase growth cone size and filopodia density that could not be

further stimulated with Netrin-1 (Menon et al., 2015). Here, the F-actin polymerase protein Ena/vasodilator stimulated phosphoprotein (VASP), which is required for Netrin-dependent increases in growth cone filopodia, was identified as a TRIM9 binding partner and could be ubiquitinated by TRIM9 (Menon et al., 2015). Netrin-1 treatment decreased ubiquitination of VASP. The filopodia increases observed in TRIM9 KO neurons were unable to be suppressed by expression of TRIM9 E3 ligase catalytic inactive mutants, DCC deficient binding, VASP deficient binding, or dimerization deficient mutants. All of these conditions also failed to rescue Netrin-1 sensitivity. Intriguingly, a competition between two E3 ligases was found to regulate VASP in an unexpected way. The RING E3 ligase TRIM67 is enriched in the cortex and cerebellum and was found to interact with DCC (Boyer et al., 2018). TRIM67 KO mice had a thinner hippocampal commissure and corpus callosum (Boyer et al., 2018). In TRIM67 KO cortical neurons, growth cones were larger and basal filopodia at growth cones were longer and insensitive to the effects of Netrin-1. These phenotypes required multiple domains of TRIM67, including its catalytic RING domain, suggesting that its ubiquitin activity was required. The lifetime of filopodia dynamics was found to be extended in TRIM67 KO neurons. Intriguingly, TRIM67 interacted with VASP, competing with TRIM9 for binding. Deletion of TRIM67 led to a surprising increase in VASP ubiquitination whereas TRIM67 overexpression decreased VASP ubiquitination without changing its steady state levels. Taken together, these studies suggested a model where netrin-dependent recruitment of TRIM67 at the tips of filopodia inhibits TRIM9-mediated ubiquitination to alleviate its constraint on filopodia formation and growth cone size. Many questions remain to be answered regarding this model that include mapping the type of ubiquitination on DCC and VASP and identification of DUBs and a TRIM67 substrate that is critical for its ability to inhibit VASP ubiquitination.

5.1.4. DUBs

DUBs, although less studied, have also been involved in regulating axon growth. Anckar et al. found that 32 DUBs were expressed in cerebellar granule neurons with different localization patterns (Anckar and Bonni, 2015). Upon RNAi-dependent depletion of individual DUBs in developing cerebellar granule neurons, depletion of USP14, USP25, USP47, and USP48 increased whereas USP4, USP7, USP20, USP21, USP25, USP27, and USP45 decreased axonal length. DUB substrates that regulate axon growth have yet to be identified.

5.2. Presynaptic differentiation and neurotransmitter release

5.2.1. Regulation of presynaptic differentiation

In Drosophila, loss-of-function of the mammalian orthologue of APC, APC2/morula resulted in an almost 2-fold increase in motor neuron synaptic boutons (van Roessel et al., 2004). APC2, along with its subunit Cdc27 were localized to presynaptic terminals at the neuromuscular junction. Upon searching for a putative APC2 substrate, Liprin-α, a protein known to regulate presynaptic development (Kaufmann et al., 2002; Zhen and Jin, 1999), contained three consensus APC binding motifs and was increased in the APC2 mutant. Removal of Liprin-α suppressed the APC2-dependent increase in presynaptic boutons suggesting that this was an APC2 substrate. Even with this presynaptic change, a loss of APC did not lead to increased quantal release but did alter postsynaptic properties by increasing postsynaptic GluRIIa. In mammals, an expressed isoform of liprin-α, liprin-α2 was found to be modulated by the proteasome and depletion of liprin-α2 using shRNA led to an increase in presynaptic bouton size and synaptic vesicle pools in cultured autaptic hippocampal neurons (Spangler et al., 2013). Interestingly, this depletion also resulted in a reduction in the expression of key presynaptic organizers that included synapsin, CASK, bassoon, Rab-interacting molecule (RIM1), piccolo, VGlut, and $Ca_V2.1$.

In cerebellar granule neurons, APC and its subunit Cdc20 were found to promote presynaptic differentiation (Yang et al., 2009). A reduction in APC^{Cdc20} by shRNA led to a decrease in synapsin and Munc13 labeled presynaptic clusters that could be rescued upon expression of a shRNA resistant form of APC^{Cdc20}. This loss also decreased presynaptic uptake as measured by the synaptic vesicle endocytosis labeling dye, FM4-64. The developmentally regulated transcription factor NeuroD2 was one possible APC^{Cdc20} substrate as it contained an APC^{Cdc20} recognition motif, was found to be ubiquitinated, and could be elevated upon proteasome inhibition. Indeed, NeuroD2 interacted with APC^{Cdc20} that required this binding motif, and RNAi-mediated knockdown of APC^{Cdc20} led to an increase in NeuroD2 expression. Overexpression of a NeuroD2 mutant that could not bind to APC^{Cdc20} reduced presynaptic synapsin clusters whereas depletion of NeuroD2 increased clusters and presynaptic uptake, which was in agreement to the contrasted phenotypes upon knockdown of APC^{Cdc20}. Collectively, these findings demonstrated that APC and its subunits function to modulate presynaptic differentiation across multiple species, using its diverse set of subunits for promotion and inhibition of presynaptic differentiation. The interplay of how APC utilizes its subunits in a regulatory manner on a temporal scale warrants further investigation.

In cerebellar granule parallel fiber axons in vivo, the RING E3 ligase RNF8 was shown to suppress presynaptic synapse formation. Removal of RNF8 increased presynaptic varicosities in the molecular layer of the cerebellar cortex that corresponded to an increase in miniature excitatory postsynaptic currents (mEPSCs) in Purkinje cell neurons (Valnegri et al., 2017). This phenotype was suggested to require cytoplasmic localization of RNF8 as expression of mutations within the nuclear export sequence but not the nuclear localization sequence of RNF8 could suppress presynaptic bouton number. Removal of the cognate E2 for RNF8, Ubc13 phenocopied the effects of RNF8 loss suggesting that these ubiquitin enzymes work in the same pathway to suppress presynaptic number. RNF8 was found to interact with another E3 ligase HERC2 and its scaffold partner NEURL4. Reduction of either HERC2 or NEURL4 increased presynaptic boutons. Expression of a shRNA resistant form of RNF8 that was unable to bind to HERC2 failed to suppress presynaptic numbers. Notably, Ubc13 is known to assemble K63-linked ubiquitin chains and HERC2 was found to coordinate with RNF8 to promote K63-ubiquitin linkages (Bekker-Jensen et al., 2010). Together, these findings suggested that a Ubc13/RNF8/HERC2 pathway suppresses presynaptic differentiation through K63-linked ubiquitination of its targets. Substrates that undergo this nonproteolytic type of modification for this pathway have yet to be identified.

5.2.2. Regulation of neurotransmitter release

Protein ubiquitination is also known to regulate glutamate release pathways. Inhibition of the proteasome increased presynaptic transmission at the glutamatergic neuromuscular junction in Drosophila (Speese et al., 2003). Treatment of cultured hippocampal neurons for 15 min or 2 h with an irreversible proteasome inhibitor increased FM4-64 uptake but did not affect vesicle release. This proteasome sensitive increase became more prominent as neurons matured in culture. Interestingly, inhibition did not alter presynaptic release suggesting a role for proteasome activity in increasing the size of the recycling vesicle pool (Willeumier et al., 2006). Acute treatment with irreversible and reversible proteasome inhibitors massively increased mEPSC frequency but did not affect mEPSC amplitudes (Rinetti and Schweizer, 2010). Interestingly, chronic presynaptic silencing of hippocampal neurons decreased the expression of priming proteins Munc13-1 and Rim1, all effects that could be blocked by MG132 (Jiang et al., 2010).

Synaptophysin is a presynaptic transmembrane protein that has been shown to regulate neurotransmitter release and vesicle recycling. The E3 ligases seven in absentia homolog 1 or 2 (Siah-1A and Siah2) were identified as binding partners in a yeast two-hybrid screen against the C-terminal tail of synaptophysin (Wheeler et al., 2002). Siah-1A was shown to increase synaptophysin ubiquitination and reduced its expression when co-expressed in nonneuronal cells. However, knockdown of Siah-1 in primary hippocampal neurons did not increase synaptophysin but did blunt hypoxia-induced degradation (Zhao et al.,

2015). Siah1 interacted with the Zn finger (ZnF) domains of Bassoon and Piccolo, major proteins involved in organizing the active zone and regulating synaptic vesicle exocytosis (Waites et al., 2013). A combination of Bassoon and Piccolo shRNAs in hippocampal neurons resulted in a decrease of major presynaptic proteins such as Munc13-1, RIM1, and synaptophysin that in part could be restored by inhibiting the ubiquitin E1, proteasome activity or expressing Bassoon or Piccolo ZnF mutants that could not bind Siah1 (Waites et al., 2013). Notably, hippocampal neurons isolated from Bassoon KO mice did not have reduced synaptophysin (Hoffmann-Conaway et al., 2020). A follow-up set of studies revealed a somewhat surprising finding in Bassoon and Piccolo ubiquitin-dependent regulation of the presynaptic active zone (Okerlund et al., 2017). Here, an increase in the number of autophagosomes was found in the presynaptic terminals of Bassoon/Piccolo shRNA hippocampal neurons and later in Bassoon KO mouse neurons (Hoffmann-Conaway et al., 2020). Like the previous study, inhibition of the E1 was able to partially suppress LC3, a protein that is enriched at autophagosomes demonstrating a requirement for protein ubiquitination in their formation. Although expression of Bassoon was also able to suppress this phenotype, shRNA-mediated knockdown of Siah1 could not. However, Siah1 shRNA knockdown did reduce LC3 that colocalized with synaptophysin1 (Hoffmann-Conaway et al., 2020). Knockdown of the E3 ligase Parkin (an E3 ligase that is mutated in rare forms of Parkinson's disease (George et al., 2018)) in Bassoon KO neurons suppressed LC3 puncta and disrupted its colocalization with Synaptophysin1 restoring synaptic vesicle pools. Another study recently identified Bassoon in a yeast 2-hybrid screen as a binding partner for the 20S proteasome core protein, Proteasome 20S Subunit Beta 4 (PSMB4) (Montenegro-Venegas et al., 2020). This mechanism was thought to be independent of the identified functions described above. Moreover, cultured Bassoon knockout neurons demonstrated a reduction in RIM1 and Munc-13. The increased proteasome activity and decrease of RIM1 and Munc-13 could be suppressed upon expression of Bassoon PSBM4-interacting regions. Taken together, these studies provide strong evidence of a multifactorial role for Bassoon in the regulation of cellular processes that control proteostasis which include proteasome, endosome, and lysosome-dependent degradation.

Parkin has further been shown to ubiquitinate multiple presynaptic proteins that include the synaptic vesicle associated proteins CDCRel-1, Synaptotagmin IV (Syt4), Synaptotagmin XI (Syt11), Endophilin-A, Syntaxin5, and α-synuclein (Chung et al., 2001; Huynh et al., 2003; Kabayama et al., 2017; Martinez et al., 2017; Trempe et al., 2009; Wang et al., 2018; Zhang et al., 2000). Alpha-synuclein is a presynaptic enriched protein that localizes to synaptic vesicles and is implicated in neurodegenerative disease (Sulzer and Edwards, 2019). Synphilin-1 binds to α-synuclein and was shown to be ubiquitinated by Parkin. Co-expression of synphilin-1 with α-synuclein increases Lewy-body-like inclusions in HEK293 cells suggesting that Parkin-dependent ubiquitination of synphilin-1 may be critical in suppressing the formation of inclusion bodies (Chung et al., 2001).

UNC-13 are a class of presynaptic membrane proteins that are well established for their ability to regulate neurotransmitter release via SNARE-dependent exocytosis (James and Martin, 2013). Treatment of Drosophila larvae NMJs with a proteasome inhibitor or genetic disruption of the proteasome increased synaptic DUNC-13 and resulted in an accumulation of a higher molecular weight product after immunoprecipitation with an anti-ubiquitin antibody (Aravamudan and Broadie, 2003; Speese et al., 2003). Fbxo45, the human orthologue of *C. elegans* FSN-1, was identified as a potential E3 ligase for UNC-13. Fbxo45 knockdown increased mEPSC frequency in cultured hippocampal neurons suggesting a presynaptic function (Tada et al., 2010). When evaluating candidate proteins, Munc-13 was found to co-immunoprecipitate with Fbxo45 and overexpression of Fbxo45 decreased Munc13-1 and reduced its half-life in nonneuronal cells. Depletion of Fbxo45 using siRNA in Neuro2a cells also led to a delay in Munc13-1 degradation; however, Fbxo45-mediated ubiquitination effects have never been tested in primary neurons (Hakim et al., 2016).

The SCF complex F-box E3 ligase SCRAPPER was found to ubiquitinate the UNC-13 presynaptic binding protein RIM1 as a means to control the releasable pool of synaptic vesicles at active zones (Yao et al., 2007). SCRAPPER has a membrane-targeting sequence, contains the cAMP-response element within its promoter, and localizes to the presynaptic terminal with synaptophysin. SCRAPPER was found to form a complex with Skp1 and Cullin, subunits of the SCF E3 complex, interacted with RIM1, and promoted RIM1 ubiquitination when immunopurified from mouse brain. SCRAPPER KO mice had increased steady state RIM1 and undetectable RIM1 ubiquitination upon inhibition of the proteasome. Interestingly, although synapse number was not altered in SCRAPPER KO mice, there was an increase in synaptic vesicle density and a decrease in docked vesicles at presynaptic terminals. Cultured hippocampal neurons from KO mice also had increases in mEPSC frequency that could be suppressed upon knockdown of RIM1. Overexpression of SCRAPPER decreased mEPSC frequency that depended on its membrane targeting or RIM1 binding domain. Increases in glutamate were also observed in multiple brain regions in SCRAPPER KO mice, among other neurotransmitters (Eto et al., 2020).

6. Postsynaptic ubiquitination

6.1. Ubiquitin-dependent control of dendritic spines

Major structures that support glutamatergic excitatory synapses are small micron sized protrusions that bud from dendrites called dendritic spines. Dendritic spines harbor key glutamatergic postsynaptic receptors at their membrane that can be anchored by scaffold proteins found in the PSD (Nimchinsky et al., 2002). Prior work has highlighted the role of key ubiquitin machinery in regulating the development and maintenance of these critical excitatory structures along with the scaffolding machinery that supports them. Not surprisingly, proteasome activity has also been found to be required for the growth of new dendritic spines (Hamilton et al., 2012).

One of the first demonstrations of protein ubiquitination in regulating dendritic spine formation was through proteasome-dependent degradation of Rap guanosine triphosphatase activating protein (SPAR), a protein that binds to PSD-95 at synapses and regulates activity dependent remodeling of dendritic spines (Pak et al., 2001). Overexpression of the activity regulated kinase Plk2 (also known as SNK) reduced the density of mature dendritic spines and increased the number of immature spines, whereas expressing a kinase defective version of Plk2 increased dendritic spine density and mature spines. Increasing synaptic activity in primary hippocampal neuron cultures led to the elevation of polo-like kinase 2 (Plk2), which was coupled to a reduction in SPAR in a manner that was proteasome dependent. (Pak and Sheng, 2003). Follow-up work also found that Plk2 was involved in the degradation of the Ras activator RasGRF1 (Lee et al., 2011).

It is well established that chemically eliciting long-term depression (LTD) with application of NMDA leads to the removal of key postsynaptic receptors and decreases dendritic spine size and density (He et al., 2011; Henson et al., 2017; Lee et al., 1998). The postsynaptic scaffold, PSD-95 was found to be degraded by the proteasome following NMDA treatment. The E3 ligase Mdm2 interacted with PSD-95 and promoted its ubiquitination. This mechanism was proposed to help facilitate the removal of AMPA receptors from the membrane to weaken synaptic strength and perhaps even allow for dendritic spine elimination (Colledge et al., 2003). Nonproteolytic ubiquitination of PSD-95 has also been proposed. Here, the E3 ligase TNF receptor-associated factor 6 (TRAF6) was found to promote K63-linked ubiquitination of PSD-95. In cultured primary hippocampal neurons, overexpression of a PSD-95 K63-ubiquitin mutant (K558) reduced its targeting to dendritic spines and prevented its ability to increase dendritic spine formation compared to wildtype. The PSD-95 ubiquitin mutant also prevented the recruitment of SPAR to postsynaptic sites in dendrites. NMDA treatment

resulted in a loss of K63-linked PSD-95, which was thought to be mediated by the DUB cylindromatosis tumor-suppressor protein (CYLD) (Ma et al., 2017). These findings suggest an intriguing interplay of PSD-95-dependent ubiquitin modifications in mediating opposing actions of dendritic spine formation, which may serve as a common theme for many synaptic proteins.

Ube3a (also known as E6-AP) is an E3 ligase that has emerged as one of the most well-studied ubiquitin constituents in the regulation of dendritic spines. Deletion of Ube3a in mice reduces dendritic spine density and length in hippocampal CA1, Layer III-V cortical neurons, basal dendrites of Layer II/III cortical neurons and Layer V neurons in visual cortex (Dindot et al., 2008; Sato and Stryker, 2010; Yashiro et al., 2009). Maintenance of dendritic spines was also shown to be affected in Ube3a-deficient neurons in Layer V of the visual cortex. Here, decreases in dendritic spine density did not emerge until the latter part of the visual critical period (postnatal day 25–29). During this time, an increase in the rate of dendritic spine elimination was also observed in Ube3a deficient neurons that further coincided with the presence of immature spines (Kim et al., 2016). Overexpression of Ube3a in Layer II/III basal dendrites increased dendritic spine density that was further increased by blocking a PKA-dependent phosphorylation site in Ube3a to render it constitutively active (Yi et al., 2015). Surprisingly, transgenic overexpression of Ube3a in mice had no effect on apical and basal dendritic spine density in Layer II cortical neurons (Smith et al., 2011). Explanations for this finding could be related to the addition of a C-terminal fusion 3xFLAG tag to Ube3a, which could disrupt its catalytic HECT activity or interaction with specific binding partners.

A potential Ube3a substrate that was proposed to regulate dendritic spine density was the RhoA-GEF, Ephexin-5 (Greer et al., 2010). Ephexin-5 knockout mice had elevated dendritic spine densities in the hippocampus that coincided with an increase in excitatory synaptic contacts. Ubiquitination of Ephexin-5 was reduced in Ube3a knockout mice and Ephexin-5 levels were found to be elevated in the Ube3a knockout mouse brain. In primary hippocampal neuron cultures, activation of EphB2 with EphrinB1 led to proteasome-dependent removal of Ephexin-5 that was Ube3a-dependent (Margolis et al., 2010). Taken together, these findings suggested that Ube3a-dependent ubiquitination of Ephexin-5 is a critical mechanism for regulating dendritic spine density.

Using a differential proteomic labeling approach called stable-isotope labeling of amino acids in mammals (SILAM), huntingtin (Htt)-associated protein (HAP1) was identified as being upregulated in Ube3a deficient mice. Here, a loss of Ube3a was found to increase autophagy in the brain and HAP1 was also shown to engage the autophagy pathway. Ube3a interacted and ubiquitinated HAP1 and blocking autophagy could rescue Ube3a-dependent decreases in dendritic spine density in cortical neuron cultures (Wang et al., 2019b). Finally, the phosphotyrosyl phosphatase activator (PTPA) was also proposed to be a Ube3a substrate to regulate dendritic spine density. Here, removal of Ube3a increased protein phosphatase 2A (PP2A) activity in the brain that emerged around the third postnatal week. PTPA was identified as being elevated in Ube3a deficient mice using SILAM and Ube3a could assemble K48-ubiquitin linkages on PTPA. Crossing Ube3a deficient mice with Ptpa haploinsufficient mice could rescue decreases in dendritic spine density in Layer 2/3 pyramidal neurons in motor and visual cortex (Wang et al., 2019a).

A suite of additional ubiquitin enzymes have been shown to regulate dendritic spine morphology through multiple pathways (Table 1). Cumulatively, the massive expansion of these enzymes in maintaining dendritic spine morphology highlights the importance of this PTM in functional aspects of excitatory synaptic development and maintenance.

6.2. Ubiquitination of glutamate receptors

6.2.1. Ubiquitination of AMPA receptors

Glutamate is the major excitatory neurotransmitter in the central nervous system and binds to glutamate receptors, which are a large group of membrane proteins that fall under the classification of ionotropic or metabotropic (Niswender and Conn, 2010; Traynelis et al., 2010; Watkins and Evans, 1981). Please see other excellent reviews in this issue for a detailed description of the classes of these receptors and their properties. Protein ubiquitination has recently emerged as a major player in the trafficking properties and stability of these receptor subunits, where α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been the major area of focus (Widagdo et al., 2017). In the mammalian system, AMPA receptors can be differentially assembled from 4 expressed subunits (GluA1-GluA4) as homo- or hetero-tetramers, mediating the majority of fast excitatory synaptic transmission in the brain. Notably, the trafficking and abundance of these receptors is critical for synaptic plasticity, a correlate for learning and memory (Diering and Huganir, 2018).

Although protein ubiquitination was detected in membrane fractions isolated from rodent brain (Chapman et al., 1994), the first direct evidence that glutamate receptor subunits were ubiquitinated came from work conducted in *C. elegans* on the non-NMDA receptor subunit glutamate receptor 1 (GLR-1). The abundance of GLR-1 in the ventral nerve cord of *C. elegans* could be regulated by ubiquitin, and ubiquitin was found to be directly conjugated to GLR-1. However, mutation of 4 lysine residues in GLR-1 abolished this ubiquitination and led to a reduction in locomotive behavior (Burbea et al., 2002), establishing a direct link between glutamate receptor ubiquitination and behavioral output. In follow-up studies, the SCF E3 F-box protein LIN-23 was found to indirectly regulate the abundance of GLR-1 through degradation of β-catenin (Dreier et al., 2005). It was later found that the DUB USP46 could reverse GLR-1 ubiquitination (Kowalski et al., 2011). At the Drosophila neuromuscular junction, inhibition of the proteasome caused an increase in the glutamate receptor subunit GluRIIB but not GluRIIA providing additional evidence of protein ubiquitination in controlling AMPA receptor subunit abundance across species (Haas et al., 2007).

6.2.2. GluA1 subunit ubiquitination

Posttranslational modifications of the C-terminal regions of AMPA receptor subunits alters channel properties, their trafficking to and from the neuronal membrane, and modulates synaptic strength (Diering and Huganir, 2018). However, early evidence in mammals suggested that AMPA receptor turnover could be regulated by proteasome activity. Indeed, the C-terminal tails of AMPA receptor subunits were identified as being ubiquitinated, with GluA1 being the first subunit identified (Lin et al., 2011; Schwarz et al., 2010; Widagdo et al., 2015). The HECT E3 ligase Nedd4 (also known as Nedd4-1) was found to be important for AMPA receptor subunit ubiquitination (Patrick et al., 2003; Zhang et al., 2009). Treatment of neuron cultures with the AMPA receptor agonist AMPA led to a selective increase in ubiquitination of GluA1 but not GluA2 or the obligatory NMDA receptor subunit NR1. This increase was dependent on Ca^{2+} signaling and AMPA receptor activity. Mutations within 4 potential ubiquitin sites (4 KR) within the GluA1 C-terminal tail prevented AMPA-mediated GluA1 internalization (known as endocytosis) but not NMDA-dependent AMPA receptor endocytosis. As predicted, increases in AMPA receptor activity also caused the E3 ligase, Nedd4-1 to move into synapses (Hou et al., 2011; Scudder et al., 2014). Nedd4-1 interacted with GluA1 and overexpression of Nedd4-1 increased GluA1 endocytosis and its subsequent trafficking to the lysosome. However, knockdown of Nedd4-1 by RNAi blocked AMPA-dependent GluA1 endocytosis (Schwarz et al., 2010). Around the same time, another study confirmed Nedd4 as an E3 ligase for GluA1 (Lin et al., 2011). Here, manipulations of Nedd4 using RNAi demonstrated its requirement for GluA1 ubiquitination. Removal of the putative Nedd4 lysine ubiquitination sites using the C-terminal 4 KR GluA1 mutant decreased internalization of GluA1, supporting the role of Nedd4-mediated ubiquitination in lysosome-dependent degradation of GluA1-containing receptors (Fig. 2). Taken together, these findings suggested that under certain conditions, GluA1 subunit ubiquitination

Table 1
List of ubiquitin enzyme genes and their known functions at glutamatergic synapses in different species. Bolded text represent homologous genes from different species.

Gene	Ubiquitin enzyme type	Organism	Function	Reference
Uba6	E1	*Mus musculus*	Positive regulator of dendritic spine density	Lee et al. (2013)
bendless	E2	*D. melanogaster*	Required for jump response/synapse formation	Thomas and Wyman (1984); Muralidhar and Thomas (1993); Oh et al. (1994)
UBE2D1 (UbcH5)	E2	*Rattus norvegicus*	Mono-ubiquitination of GluA2 for lysosome-dependent degradation	Ghilarducci et al. (2021)
UBE2N (UbcH13)	E2	*Rattus norvegicus*	K63-linked ubiquitination of GluA2 for lysosome-dependent degradation	Ghilarducci et al. (2021)
Cdc20	E3	*Rattus norvegicus*	Positive regulator of presynaptic differentiation	Yang et al. (2009)
Cdh1	E3	*Rattus norvegicus*	Control of axon growth, Homeostatic control via GluA1 ubiquitination	Konishi et al. (2004); Fu et al. (2011)
Cul3	E3	*Rattus norvegicus*	GluK2 ubiquitination	Marshall et al. (2011)
Fbxo2	E3	*Rattus norvegicus*	GluN1 ubiquitination	Kato et al. (2005)
(*Fbxo45*) FSN-1	E3	*C. elegans*	Axon overgrowth	Bloom et al. (2007)
Fbxo45	E3	*Mus musculus*	Radial migration, axon midline crossing	Saiga et al. (2009); Tada et al. (2010)
Herc1	E3	*Mus musculus*	Dendritic spine maturation	Perez-Villegas et al. (2018)
Herc2	E3	*Rattus norvegicus*	Suppressor of presynaptic synapse formation	Valnegri et al. (2017)
Hrd-1	E3	*Rattus norvegicus*	Negative regulator of dendritic spine density	Saldate et al. (2018)
lin-23	E3	*C. elegans*	Outgrowth of mechanosensory neurons/AMPA receptor abundance	Mehta et al. (2004); Burbea et al. (2002)
Mdm2	E3	*Rattus norvegicus*	Dendritic spine elimination and AMPA receptor removal	Colledge et al. (2003)
Mib1	E3	*Rattus norvegicus*	Negative regulator of dendritic spine density	Mertz et al., 2015
Mib2	E3	*Rattus norvegicus*	GluN2B ubiquitination	Jurd et al. (2008)
morula	E3	*D. melanogaster*	Negative regulator of synaptic boutons, increase in GluRIIa	van Roessel et al. (2004); Spangler et al. (2013)
*Mylip (*IDOL*)*	E3	*Mus musculus*	Spine morphogenesis	Gao et al. (2017)
nedd4	E3	*D. melanogaster*	Axon midline crossing	Myat et al. (2002)
Nedd4	E3	*Rattus norvegicus*	K63-linked ubiquitination of GluA1 for lysosomal degradation, ubiquitination of GluN2D, ubiquitination of mGluR7	Patrick et al. (2003); Zhang et al. (2009); Gautam et al. (2013); Lee et al. (2019)
Nedd4-2	E3	*Mus musculus*	Control of neural excitability via GluA1 ubiquitination	Jewett et al. (2015); Zhu et al. (2017)
Neuralized	E3	*Mus musculus*	Positive regulator of dendritic spine density	Pavlopoulos et al. (2011)
Parkin	E3	*Rattus norvegicus*	GluN1 and GluK2 ubiquitination	Zhu et al. (2018); Maraschi et al. (2014)
Praja2	E3	*Rattus norvegicus*	Degrades PKA R subunits to regulate LTP	Lignitto et al. (2011)
(*Phr*) *rpm-1*	E3	*C. elegans*	Regulates vesicle assembly	Schaefer et al. (2000)
(*Phr*) *highwire*	E3	*D. melanogaster*	Axon growth and presynaptic bouton size	Wan et al. (2000)
(Phr) esrom	E3	*D. rerio*	Axon target selection and axon midline crossing	D'Souza et al. (2005); Hendricks et al. (2008)
(Phr) Phr	E3	*Mus musculus*	Axon innervation and midline crossing	Lewcock et al. (2007)
Rnf8	E3	*Rattus norvegicus*	Suppressor of presynaptic synapse formation	Valnegri et al. (2017)
Rnf167	E3	*Rattus norvegicus*	Ubiquitination of GluA2 for lysosomal degradation	Lussier et al. (2012)
Scrapper	E3	*Mus musculus*	Regulates releasable poool of synaptic vesicles at active zones	Yao et al. (2007)
Siah-1A	E3	*Rattus norvegicus*	Formation of presynaptic active zone, ubiquitination of mGlur1a and mGluR5	Waites et al. (2013); Ishikawa et al., 1999; Moriyoshi et al. (2004)
Traf6	E3	*Mus musculus, Rattus norvegicus*	Dendritic spine formation	Ma et al. (2017)
Trim3	E3	*Mus musculus*	Negative regulator of dendritic spine density	Hung et al. (2010); Schreiber et al. (2015)
(*Trim9*) *madd-2*	E3	*C. elegans*	Axon branching and guidance	Hao et al. (2010)
(Trim9) asap	E3	*D. melanogaster*	Axon branching and guidance in sensory neurons	Morikawa et al. (2011)
Trim9	E3	*Mus musculus*	Axon branching and guidance	Winkle et al. (2014)
Trim67	E3	*Mus musculus*	Regulates axon growth cone size	Boyer et al. (2018)
Ube3a	E3	*Mus musculus*	Dendritic spine formation/turnover	Dindot et al. (2008); Kim et al. (2016)
Ube3b	E3	*Mus musculus*	Negative regulator of dendritic spine density	Ambrozkiewicz et al. (2020)
Ap-Uch	DUB	*Aplysia californica*	Required for 5-HT induced LTF	Hegde et al. (1997)
A20	DUB	*Mus musculus*	Negative regulator of dendritic spine density and size	Mei et al. (2020)
fat facets	DUB	*D. melanogaster*	Negatively controls presynaptic size	DiAntonio et al. (2001)
Uch-l1	DUB	*Rattus norvegicus*	Regulator of dendritic spine density and size	Cartier et al. (2009)
USP4	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
USP6	DUB	*Mus musculus*	GluN1 de-ubiquitination	Zeng et al. (2019)
USP7	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
USP8	DUB	*Rattus norvegicus*	AMPA receptor recycling, promotes dendritic spine stability	Scudder et al. (2014); Kerrisk Campbell and Sheng, 2018
Usp9x	DUB	*Mus musculus*	Positive regulator of dendritic spine density and size	Yoon et al. (2020)
USP14	DUB	*Rattus norvegicus*	Negative regulator of axon growth	Anckar and Bonni (2015)
USP20	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
USP21	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
USP25	DUB	*Rattus norvegicus*	Negative regulator of axon growth	Anckar and Bonni (2015)
USP27	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
USP45	DUB	*Rattus norvegicus*	Positive regulator of axon growth	Anckar and Bonni (2015)
usp46	DUB	*C. elegans*	GLR-1 abundance	Kowalski et al. (2011)
Usp46	DUB	*Rattus norvegicus*	GluA1 abundance	Huo et al. (2015)
USP47	DUB	*Rattus norvegicus*	Negative regulator of axon growth	Anckar and Bonni (2015)
USP48	DUB	*Rattus norvegicus*	Negative regulator of axon growth	Anckar and Bonni (2015)

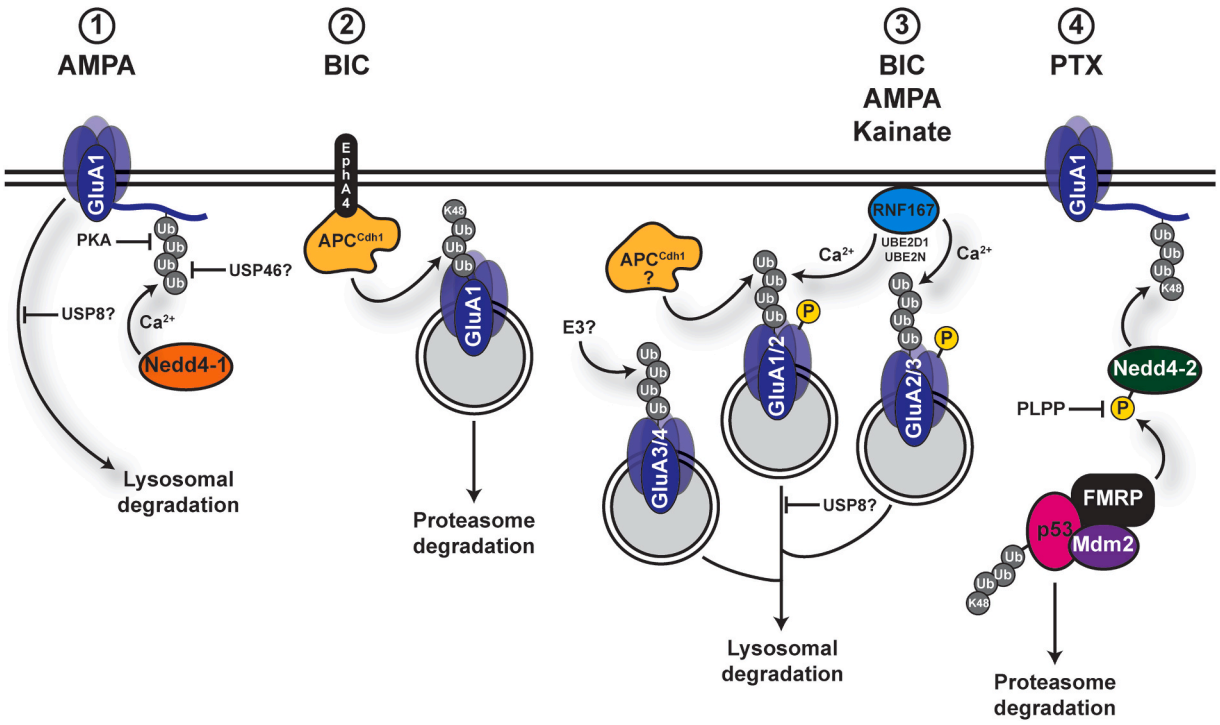

Fig. 2. Ubiquitination of AMPA receptor subunits. Multiple proposed mechanisms of AMPA receptor ubiquitination. GluA1-containing AMPA receptor subunits can be ubiquitinated (Ub) by multiple pathways. 1.) AMPA stimulation leads to ubiquitination of GluA1 subunits by the E3 ligase Nedd4-1. This pathway requires Ca^{2+} signaling and is thought to lead to lysosome-dependent degradation of GluA1. This may be counteracted by the DUBs, USP46 or USP8 and PKA phosphorylation of the GluA1 C-terminal tail at Ser-845. 2.) Treatment of neurons with bicuculline (BIC) leads to recruitment of APC^{Cdh1} to EphA4 which stimulates K48-linked ubiquitination of GluA1. This process leads to proteasome-dependent GluA1 degradation. 3.) BIC, AMPA, or kainate stimulation lead to ubiquitination of all AMPA receptor subunits and phosphorylation of GluA2 on Ser-880. The E3 ligase RNF167 coordinates with the E2s UBE2D1 and UBE2N to promote the ubiquitination of GluA2 AMPA receptor subunits. These pathways are thought to lead to lysosome-dependent degradation. 4.) Treatment of neurons with picrotoxin (PTX) engages Mdm2 dependent ubiquitination of p53 and forms a complex with FMRP. FMRP is required for Nedd4-2 phosphorylation which ubiquitinates GluA1 via K48-linkages to promote proteasome-dependent degradation of GluA1. This process can be blocked by the phosphatase, PLPP.

by Nedd4 traffics AMPA receptors for their subsequent degradation by the lysosome.

Protein kinase A (PKA)-dependent phosphorylation of the GluA1 C-terminal tail at Serine 845 has been shown to promote receptor recycling (Ehlers, 2000; Roche et al., 1996) and was recently found to modulate GluA1 ubiquitination (Guntupalli et al., 2017). Here, creation of a phosphomimetic mutant at Ser-845 reduced GluA1 ubiquitination and further decreased its interaction with Nedd4. Thus, phosphorylation of Ser-845 most likely serves to antagonize GluA1 ubiquitination and promote recycling or retention at the membrane (Guntupalli et al., 2017). In these studies, the type of ubiquitin that was added to GluA1 was never determined. The addition of mono- and K63- ubiquitin are thought to be the predominant linkages that function in receptor trafficking and lysosome degradation (Piper et al., 2014) although there is evidence for other linkages to participate in this process (Locke et al., 2014; Sliter et al., 2011; Zemoura et al., 2013). The type of ubiquitination on GluA1 was partially resolved by Widagdo et al. where K63-linked ubiquitin chains were found to assemble on GluA1 (Widagdo et al., 2015).

As stated earlier, protein ubiquitination is a reversible process where the presence of DUBs remove and edit ubiquitin moieties (Komander et al., 2009). Knockdown of the DUB USP46 in cortical neurons led to an accumulation of GluA1 ubiquitin conjugates, suggesting that like in *C. elegans* (Kowalski et al., 2011), USP46 is a major DUB for GluA1. USP46 overexpression was able to decrease the turnover of GluA1 and reduce its internalization (Huo et al., 2015). Another DUB, USP8 was suggested to remove GluA1 ubiquitination triggered by AMPA treatment as a means to promote AMPA receptor recycling instead of degradation in neurons (Scudder et al., 2014) (Fig. 2).

The HECT family member of Nedd4, Nedd4-2 was also found to ubiquitinate the GluA1 subunit (Jewett et al., 2015; Zhu et al., 2017) requiring the same 4 lysines (4 KR) as for Nedd4 (Lin et al., 2011; Schwarz et al., 2010). Nedd4-2 promoted GluA1 ubiquitination when overexpressed in HEK293 cells. In neurons, treatment with the $GABA_A$ antagonist picrotoxin (PTX) increased Nedd4-2 and reduced GluA1. Deletion of Nedd4 isoform 1 in mice blocked PTX-induced GluA1 ubiquitination, which could also be decreased upon inhibition of the E3 ligase for p53, Mdm2 or with blocking the interaction between p53 and Mdm2 with the small molecular inhibitor Nutlin-3 (Jewett et al., 2015). A loss of isoform 1 of Nedd4-2 further resulted in increased excitatory spontaneous neurotransmission in cortical neuron cultures that was coupled to an increase in surface GluA1. This observed increase in excitation could be blunted upon genetic reduction of GluA1 (Zhu et al., 2017). Intriguingly, missense mutations in Nedd4-2 cause epilepsy (George et al., 2018) and expression of these mutants dramatically decreased GluA1 ubiquitination, suggesting that epilepsy mutations lead to derangements in GluA1 ubiquitination which could explain the increased excitability in these individuals (Zhu et al., 2017). A potential mechanism of Nedd4-2 control of GluA1 ubiquitination was proposed to be mediated by phosphorylation of Nedd4-2. Nedd4-2 was found to be critical for homeostatic synaptic downscaling requiring the RNA binding factor fragile X mental retardation 1 (FMR1) (Lee et al., 2018). Here, PTX-induced ubiquitination of GluA1 in cortical neuron cultures was absent in Fmr1 KO mice and was coupled to a significant decrease in Nedd4-2 but not Nedd4-1 GluA1 binding. PTX also increased the phosphorylation of Nedd4-2 at Serines 342 and 448; however, the opposite effect was observed in cultured cortical neurons from Fmr1 KO mice. Mutations of Ser-342 and Ser-348 to alanine decreased GluA1-Nedd4-2 interaction and GluA1 ubiquitination in HEK293 cells. Interestingly, overexpression of Nedd4-2 or inhibition of p53 with pifithrin-α could

restore PTX-induced GluA1 ubiquitination in Fmr1 KO mice as well as PTX-induced synaptic downscaling. The pyridoxal-5′-phosphate phosphatase/chronophin (PLPP/CIN) phosphatase was found to dephosphorylate Nedd4-2. PLPP/CIN KO mice had increased Nedd4-2 and a reduction in GluA1 ubiquitination (Kim et al., 2019). These findings suggested that dephosphorylation of Nedd4-2 at Ser-448 by PLPP/CIN after seizure, reduces Nedd4-2 activity, which in turn decreases GluA1 ubiquitination leading to hyperexcitability. The phosphorylation of Nedd4-2 is controlled by activity-dependent pathway interactions between FMRP, Mdm2, p53, and 14-3-3 (Fig. 2). Determining if Nedd4-2 disease point mutations have reduced phosphorylation of Ser-448 and enhanced interactions with PLPP/CIN would further support this model.

In another study, the GluA1 subunit was found to be ubiquitinated by a different mechanism at its N-terminal extracellular domain (Fu et al., 2011). Here, GluA1 ubiquitination was suggested to be an essential step for mediating homeostatic plasticity. Increasing network activity with prolonged treatment of bicuculline led to a reduction in synaptic GluA1 and an increase in the activation of the tyrosine kinase ephrin type-A receptor 4 (EphA4). This activity-induced downregulation of GluA1 was proteasome dependent. The modular E3 ligase subunit of APC, Cdh1 was found to interact with both EphA4 and GluA1. This complex interaction led to polyubiquitination of the GluA1 subunit with a suggested -K48 ubiquitin linkage assembly. Interestingly, the N-terminal region of GluA1 contains 3 consensus APC degradation motifs that consist of two D-boxes and one A-box (Davey and Morgan, 2016). Mutation of the D-box, but not the A-box motif prevented APC^{Cdh1}-overexpression induced loss of GluA1 indicating that these sites were required for GluA1 degradation. Expression of the GluA1 4 KR mutant used by Schwarz et al. did not block APC^{Cdh1}-induced GluA1 degradation suggesting that alternative sites within GluA1 were targeted for ubiquitination (Fig. 2). Although modulation of APC^{Cdh1} prevented bicuculline-induced downscaling of AMPA receptor mEPSC amplitudes, the role of APC^{Cdh1}-selective actions on GluA1 in homeostatic plasticity has yet to be established (Fu et al., 2011).

Notably, GluA1 ubiquitination may be implicated in neurological conditions such as Alzheimer's disease (AD) and chronic stress. AD is characterized by alterations in AMPA receptor endocytosis and a decrease in surface AMPA receptors which are thought to be driven in part by excess Aβ oligomers (Guntupalli et al., 2016). Indeed, addition of Aβ oligomers reduced GluA1 phosphorylation at Ser-845 (Minano-Molina et al., 2011) and increased GluA1 ubiquitination at lysine 868 (Guntupalli et al., 2017). This resulted in decreased AMPA receptor surface expression. However it was not established if endocytosis was a prerequisite for GluA1 ubiquitination and if this required Ca^{2+} and NMDA receptor activity (Guntupalli et al., 2017). Although the E3 ligase for this ubiquitination was not identified, another study found that Nedd4 was required for Aβ oligomer-induced AMPA receptor removal (Rodrigues et al., 2016). It is well established that stress alters glutamatergic signaling in the prefrontal cortex (PFC) (Yuen et al., 2017). Under conditions of repeated stress, surface AMPA and NMDA receptor subunits were decreased in PFC. This effect was dependent on glucocorticoid receptor (GR) and proteasome activity as addition of the GR antagonist RU486 or the proteasome inhibitor MG132 blocked this loss. In addition to a reduction, an increase in GluA1 ubiquitination was also detected in the PFC, which could also be blocked by RU486. Nedd4 was found to be responsible for stress-induced GluA1 ubiquitination as RNAi-mediated knockdown of Nedd4 blocked stress-induced ubiquitination of GluA1 (Yuen et al., 2012). Cumulatively, these studies highlight a potential role for Nedd4-dependent AMPA receptor ubiquitination in the pathogenesis leading to stress and neurodegenerative disease.

6.2.3. GluA2 subunit ubiquitination

The GluA2 AMPA receptor subunit has also been identified as a target for ubiquitination. GluA2 ubiquitination was detected after increasing neuronal excitation with bicuculline, kainate or AMPA, and required Ca^{2+} and NMDA receptor activity whereas stimulation with NMDA or the group I mGluR agonist, DHPG did not promote GluA2 ubiquitination (Lussier et al., 2011). The activity-induced increase in GluA2 ubiquitination could be blunted by inhibiting clathrin-dependent endocytosis with the dynamin inhibitor dynasore demonstrating that endocytic pathways are required for AMPA receptor ubiquitination. Using a screen to identify regulators of surface GluA2-containing AMPA receptors, three RING finger E3 ligases were identified (RNF112, RNF144, and RNF167) where RNF167 had the greatest effect (Lussier et al., 2012). RNF167 was found to predominantly localize at endosomes and lysosomes in neurons, promoting GluA2 ubiquitination in a manner that was dependent on its E3 ligase activity. Moreover, bicuculline-induced GluA2 ubiquitination could be reduced in RNF167 depleted neurons. A decrease in RNF167 increased surface GluA1, GluA2, and GluA3 AMPA receptor subunits and enhanced AMPA receptor currents in hippocampal neurons. In a follow-up study, RNF167 was found to directly bind to the C-terminus of GluA2 in vitro. However, the ability of RNF167 to ubiquitinate GluA2 required the coordination of two separate E2 enzymes, UBE2D1 (also known as UbcH5) and UBE2N (also known as UbcH13). Both of these E2s colocalized at endosomes and lysosomes in dendrites. In vitro ubiquitin assays demonstrated that UBE2D1 served as a priming enzyme to mono-ubiquitinate GluA2 for subsequent UBE2N-dependent -K63-linked ubiquitination (Ghilarducci et al., 2021). An intriguing model is that increased activity leads to GluA2 mono-ubiquitination by UBE2D1/RNF167, which shuttles GluA2 to the lysosome where UBE2N/RNF167-dependent ubiquitination promotes lysosome-dependent degradation of GluA2 to attenuate AMPA-mediated excitatory synaptic transmission (Fig. 2).

In another study, bicuculline-induced GluA2 ubiquitination was mapped to Lysines 870 and 882 found within the C-terminal tail of GluA2 (Widagdo et al., 2020). Here, another interesting relationship between receptor phosphorylation and ubiquitination was identified, but unlike GluA1, the modifications appeared to be synergistic. Protein kinase C (PKC) activation increases the phosphorylation of GluA2 at Serine 880 and facilitates its endocytosis (Chung et al., 2000). Interestingly, treatment with the PKC activator phorbol ester (PMA) also promoted GluA2 ubiquitination, suggesting a positive relationship between GluA2 phosphorylation and its ubiquitination. Indeed, co-application of bicuculline and PMA further enhanced GluA2 ubiquitination then when treated alone. Creation of a GluA2 Ser-880 phosphomimetic enhanced bicuculline-induced GluA2 ubiquitination (Fig. 2). One perplexing aspect of this study is that Ser-880 is sufficient but not required for PMA-induced bicuculline augmentation of GluA2 ubiquitination as mutation of Ser-880 to alanine did not block PMA-induced augmentation. One possible explanation is that PMA might activate the E3 ligase for GluA2, RNF167 (Widagdo et al., 2020).

6.2.4. Coordinated actions of AMPA receptor subunit ubiquitination

All 4 AMPA receptor subunits were found to be ubiquitinated following treatment with bicuculline as well as AMPA (Widagdo et al., 2015). This finding was in contrast to the selective ubiquitination of GluA1 observed by Schwarz et al. and selective ubiquitination of GluA2 observed by Lussier et al. The types of antibodies used in these studies were targeted toward a different region of the protein and conditions for ubiquitin detection were performed using different buffers and denaturing conditions, which may in part explain the discrepancy in results. However, similar to all of these studies, AMPA receptor subunit ubiquitination was found to be NMDA and Ca^{2+} dependent, requiring ubiquitination of the C-terminal tails of GluA1 and GluA2. Interestingly, activation of mGluRs with the group I agonist, DHPG did not lead to AMPA receptor ubiquitination. Blocking clathrin-dependent endocytosis with dynasore resulted in a loss of activity-induced AMPA receptor ubiquitination of all subunits. Cumulatively, this led to a model where AMPA receptors were ubiquitinated after their removal from the membrane and further supported the role of this PTM in regulating the intracellular sorting of AMPA receptor cargo into late endosomes

(Fig. 2). The role of GluA3 and GluA4 subunit ubiquitination has yet to be established.

6.3. Ubiquitination of NMDA receptors

NMDA receptors are hetero-tetrameric proteins that bind glutamate and are differentially assembled using three subunits, GluN1, GluN2 (A-D), and GluN3 (Traynelis et al., 2010). Although not as well studied, NMDA receptor subunits can be directly ubiquitinated (Lussier et al., 2015).

6.3.1. GluN1 subunit ubiquitination

The E3 ligase SCF complex substrate specific subunit, Fbxo2 was found to interact with glycosylated GluN1 and this interaction was mediated through its F-box associated domain (FBA) (Kato et al., 2005). Interestingly, Fbxo2 is associated with endoplasmic reticulum associated degradation (ERAD), a type of pathway that removes orphan subunits and misfolded proteins that are transported out of the endoplasmic reticulum (ER) (Yoshida et al., 2019). Fbxo2 increased ubiquitination of GluN1 in HEK293 cells. Additionally, in neurons, an increase in GluN1 ubiquitination was observed following bicuculline treatment. Deletion of the FBA domain of Fbxo2 resulted in an increase in extrasynaptic NMDA receptor currents (Kato et al., 2005). Fbxo2 knockout mice showed an increase in membrane localized GluN1 and GluN2A starting at 3 months of age with no changes in GluN2B (Atkin et al., 2015). Surface removal of GluN1 was blocked upon increasing NMDA receptor internalization with bicuculline in Fbxo2 knockout mouse neurons. Interestingly, although surface GluN1 and GluN2 were elevated in the hippocampus, this did not lead to changes in NMDA receptor-mediated synaptic currents or long-term potentiation, nor did it alter dendritic spine densities (however dendritic spine length and the formation of axo-dendritic synapses were increased).

Fbxo2 drives lysosome-phagosome mediated removal of GluN1, a process critical for regulating the steady-state pool of NMDA receptors. GluN1 was found to be ubiquitinated in nonneuronal cells and this ubiquitination could be blocked by addition of the cytoskeleton-associated GluN1 binding partner, neurofilament light (NF-L) chain (Ehlers et al., 1998; Ratnam and Teichberg, 2005). The addition of NF-L also increased surface GluN1 indicating that NF-L blocks ubiquitin-mediated degradation of GluN1 by anchoring NMDA receptors at the membrane. In these studies, the type of ubiquitin modification and the mapping of ubiquitination sites for GluN1 were never established. It would be intriguing to determine if NF-L anchoring of NMDA receptors antagonizes GluN1-mediated trafficking. Indeed, deletion of NF-L in mice resulted in a reduction in GluN1, similar to NMDA receptor hypofunction observed in schizophrenia (Yuan et al., 2018). This reduction in GluN1 was associated with an increase in K48-linked GluN1 ubiquitination, which is consistent with the type of modification mediated by Fbxo2. These findings supported a putative model whereby NF-L anchors NMDA receptors to antagonize their ubiquitination of GluN1 by Fbxo2. Fbxo2 ubiquitination of GluN1 drives lysosome-phagosome mediated removal of GluN1, a process critical for regulating the steady-state pool of NMDA receptors (Fig. 3). Future studies crossing NF-L with Fbxo2 knockout mice or reducing Fbxo2 in NF-L KOs would provide additional evidence for such a mechanism. GluN1 ubiquitination was also observed in the PFC after exposure of mice to repeated stress, which was blocked by depletion of Fbxo2 using RNAi or the addition of the glucocorticoid receptor antagonist RU486 or MG132 (Yuen et al., 2012). Collectively, these findings provided the first evidence that an E3 member of the ERAD pathway can regulate glutamate receptors.

An additional E3 ligase was found to ubiquitinate GluN1. Here, overexpression of the E3 ligase Parkin selectively promoted GluN1 ubiquitination and human disease-associated point mutations prevented this effect (Zhu et al., 2018). However, upon RNAi-mediated knockdown of parkin, there was a reduction in surface GluN1 and decreased excitatory neurotransmission. The Parkin effects on regulating surface glutamate receptors and excitatory neurotransmission are in contrast to other studies which actually found an increase (Cremer et al., 2015; Helton et al., 2008). Nevertheless, this perplexing finding suggests that ubiquitination of GluN1 by Parkin might stabilize surface GluN1 or perhaps regulate the forward trafficking or recycling of NMDA receptors (Fig. 3).

6.3.2. GluN2B subunit ubiquitination

Mind bomb-2 (Mib2) is an E3 ligase that is enriched at postsynaptic sites and was identified as a binding partner in a yeast three-hybrid screen to capture Fyn (a tyrosine kinase) phosphorylation-dependent GluN2B complexes (Jurd et al., 2008). Mib2 overexpression increased ubiquitination of GluN2B, an effect that could be modulated by Fyn-dependent phosphorylation of NR2B and could be ablated upon removal of the Mib2 ZnF GluN2B interacting domain. In HEK293 cells, co-expression of Mib2 with the complement of NMDA receptor subunits resulted in a decrease in currents that was dependent on the Mib2 ZnF domain and proteasome activity. However, although generation of *Mib2* KO mice showed alterations in hippocampal-dependent learning tasks and impairments of theta burst stimulation-induced early long-term potentiation (E-LTP) and early long-term depression (E-LTD), they did not exhibit impairments in baseline excitatory neurotransmission, NMDA receptor-dependent LTD, or total GluN2B (Kim et al., 2015).

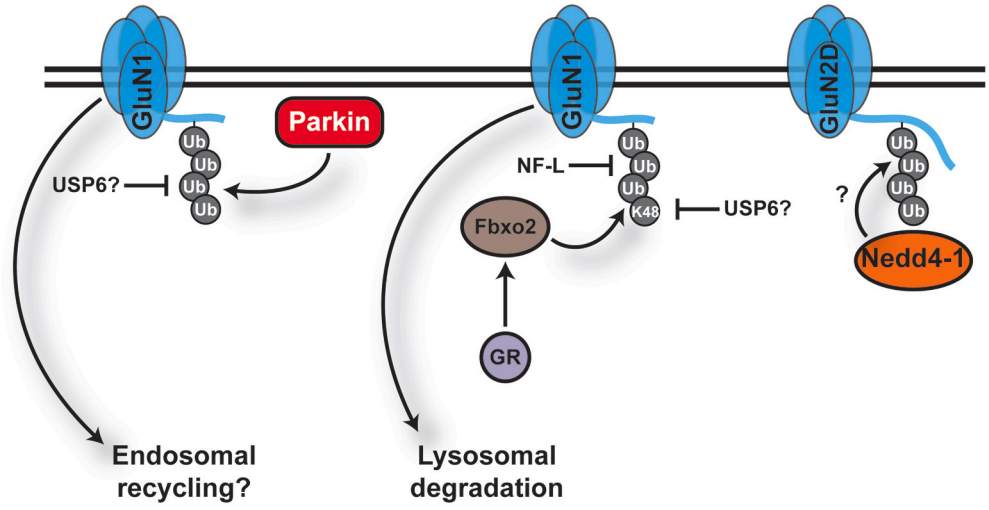

Fig. 3. Ubiquitination of NMDA receptor subunits. Left, Parkin can ubiquitinate (Ub) GluN1-containing NMDA receptor subunits, which may promote endosomal recycling. This process may be counteracted by the DUB, USP6. Middle, activation of the glucocorticoid receptor leads to Fbxo2-dependent ubiquitination of GluN1 and assembles K48-linkages to promote lysosome-dependent degradation. These effects can be inhibited by NF-L or the DUB USP6. Right, Nedd4-1 may ubiquitinate the GluN2D receptor.

These findings suggested that another Mib2 substrate may cause plasticity and learning impairments. Notch ligand Delta could be a potential substrate considering it was the first Mib2 substrate identified (Itoh et al., 2003) and a decrease in cleaved Notch1 was observed in *Mib2* KO mice 1 h after receiving a mild footshock. One caveat to this study is that GluN2B ubiquitination, synaptic localization and activity-induced alterations were never measured in these KO mice. Thus, it is difficult to unequivocally rule out the possibility of alterations in GluN2B ubiquitination in *Mib2* KOs (Kim et al., 2015).

6.3.3. GluN2D subunit ubiquitination

Using a protein-binding mass spectrometry approach targeting the C-terminal regions of GluN2D, the E3 ligase Nedd4 was identified as a binding partner (Gautam et al., 2013). Co-expression of Nedd4 with GluN2D increased GluN2D ubiquitination in HEK-293T cells and reduced GluN1/GluN2D receptor currents when expressed in Xenopus oocytes in a manner dependent on the Nedd4 catalytic HECT domain (Fig. 3). In all of these studies, establishing the role of these ubiquitination sites in regulating GluN1 trafficking and synaptic localization would help determine if protein ubiquitination is a universal theme for the trafficking of glutamate receptors.

6.3.4. DUBs and NMDA subunit de-ubiquitination

Like AMPA receptors, DUBs have been identified in removing NMDA receptor ubiquitination. Transgenic overexpression of the human DUB USP6, led to learning and memory enhancements and increased social behavior in mice (Zeng et al., 2019). Moreover, hippocampal NMDA-dependent LTP was enhanced in these transgenic mice. Total and synaptic GluN1, GluN2A, and GluN2B were increased and, GluN1 and GluN2B were found to bind to USP6 in brain extracts. USP6 transgenic mice had reduced GluN1 ubiquitination and depletion of USP6 in human embryonic stem cells differentiated into excitatory neurons resulted in a reduction in surface GluN1 leading to a model that the presence of USP6 stabilizes NMDA receptors perhaps through promoting GluN1 recycling to the membrane (Fig. 3).

6.4. Ubiquitination of kainate receptors

Kainate receptors are the last class of ionotropic glutamate receptors and are heteromers that comprise of five subunits, GluK1-GluK5 (Traynelis et al., 2010). When using the C-terminus of GluK2 as bait, the BTB (from bric a brac, tramtrack, and broad complex)/Kelch repeat domain protein actinfilin was identified as a binding partner (Marshall et al., 2011). Interestingly, BTB domain containing proteins can bind to Cul3, a core scaffold protein which is assembled into a multisubunit Cullin-RING E3 ligase complex (Lydeard et al., 2013). Overexpression of actinfilin decreased GluK2 and could be restored after blocking proteasome activity with MG132. Cul3 heterozygous mice were found to have elevated synaptic GluK2 and reduced ubiquitination. RNAi-mediated knockdown of actinfilin or expression of a dominant negative version of Cul3 increased surface GluK2 at similar magnitudes in primary hippocampal neurons. These findings suggest that actinfilin serves as a scaffold for Cul3 complex-based ubiquitination of GluK2.

GluK2 was found to be significantly increased in the substantia nigra of a transgenic mouse that expresses a human variant of parkin that causes autosomal recessive juvenile parkinsonism (Q311X) (Maraschi et al., 2014). Using quantitative autoradiography, increases in kainate receptor density were observed in motor, somatosensory, piriform, and the visual cortices of Parkin and DJ-1 (DJ-1 is a gene that is linked to autosomal-recessive Parkinson's disease) KO mice but increases in the substantia nigra were only found in DJ-1 KO mice (Cremer et al., 2015). Moreover, increases in GluK2 were observed in frontal cortex brain lysates from four separate PARK2 patients. Parkin interacted and promoted ubiquitination of GluK2 in HEK293 cells and depletion of Parkin using shRNA increased surface levels, decreased ubiquitination, and increased kainate currents in hippocampal neurons, which could be rescued by expression of a shRNA-resistant form of Parkin (Maraschi et al., 2014). Taken together, these findings suggested deficits in Parkin-mediated GluK2 ubiquitination may lead to excitotoxicity in Parkinson's individuals with PARK2 mutations.

6.5. Ubiquitination of mGluRs

Metabotropic glutamate receptors are G-protein coupled receptors that are divided into three families consisting of group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), or group III (mGluR4 and mGluR6-8) receptors (Niswender and Conn, 2010). To date, only group I and III receptors have been identified as being ubiquitinated.

6.5.1. Ubiquitination of group I mGluRs

The RING E3 ligase Seven in abstentia homolog 1 (Siah1A) was identified as a binding partner of the C-terminal tail of group I mGluRs. Interaction between Siah-1A and mGluR5a required its Ca^{2+}/calmodulin (CaM) binding site and increases in Ca^{2+} decreased Siah1A binding with the mGluR5a C-terminus while increasing binding to CaM. These findings suggested a competition between Siah1A and CaM (Ishikawa et al., 1999). Although overexpression of Siah1A in superior cervical ganglion (SCG) sympathetic neurons did not alter the clustering or surface mGluR5a, it did decrease glutamate induced Ca^{2+} currents when co-expressed with group I mGluRs, with no effect on the group II mGluR, mGluR2. The Siah1A effect on mGluR-associated currents could still be blocked by removing the catalytic RING domain of Siah1A and was blunted by co-expression of CaM suggesting that the Siah1A effect on group I mGluRs was binding and not ubiquitin dependent (Kammermeier and Ikeda, 2001). The studies above provided evidence that group I mGluRs could be regulated by Siah1A but they did not directly test Siah1A-dependent stability or ubiquitination. This was resolved in part by overexpression studies in nonneuronal cells where expression of Siah1A was found to ubiquitinate and decrease mGluR1α and mGluR5 but not mGluR3 or mGluR7 (Moriyoshi et al., 2004). This loss of mGluR1α and mGluR5 could be rescued after treatment with MG132. The reduction in mGluR1α was also dependent on the Siah1A RING domain and the mGluR1a Siah1A interacting domain.

A more detailed model of the relationships between CaM and Siah1A binding was provided in a study that tested the role of a PKC-dependent phosphorylation site found within the C-terminal tail of mGluR5 which was known to disrupt binding of CaM to mGluR5 (Lee et al., 2008). Here, Siah1A was also found to decrease mGluR5 and creation of a mGluR5 phosphomimetic at Serine 901 decreased mGluR5 stability in HeLa cells (Ko et al., 2012). This instability could be restored following RNAi-mediated reduction of Siah1A. The mGluR5 phosphomimetic also preferentially interacted with Siah1A as opposed to CaM. Unlike the previous study (Ishikawa et al., 1999), overexpression of Siah1A in HeLa cells dramatically reduced surface mGluR5 that was dependent on its RING E3 ligase activity. Overexpression of Siah1A also decreased surface mGluR5 in cultured hippocampal neurons (Ko et al., 2012). Finally, details on how group I mGluR trafficking was modulated by ubiquitination and its link to AMPA receptor trafficking was elucidated in a study by Gulia et al. (2017). Here, activation of mGluRs by the group I agonist DHPG increased mGluR1 K63-linked ubiquitination and endocytosis of mGluR1 and mGluR5 when expressed in HEK293 cells and overexpressed in cultured hippocampal neurons. These effects were dependent on protein ubiquitination as they could be blocked by an irreversible inhibitor of the ubiquitin E1 PYR-41. The effects on DHPG-induced mGluR1 ubiquitination and endocytosis could be blunted upon knockdown of Siah1A using siRNA. As expected, the loss of Siah1A also resulted in enhanced endocytosis of GluA1-containing AMPA receptors. Cumulatively, these findings provided a model whereby stimulation of group I mGluRs leads to PKC-dependent phosphorylation of mGluR5, dissociation of mGluR5 from CaM to allow for Siah1A-dependent binding and ubiquitination of group I mGluRs, which in turn stimulates their endocytosis. This model provides some type of

mechanistic explanation for the interplay between group I mGluR trafficking and AMPA receptor endocytosis (Citri et al., 2009). However, CaM does not bind to mGluR1 so details on how it is ubiquitinated by Siah1A are still lacking (Choi et al., 2011). Moreover, the functional significance of this mechanism on synaptic plasticity and associated behaviors is implied but has yet to be firmly established. Regardless, these studies provided evidence that Siah1A serves as a group I mGluR brake, to limit the effects of overstimulation and AMPA receptor endocytosis (Fig. 4).

Although mGluR1 ubiquitination was shown to be K63-linked, a function for proteasome-dependent mGluR1α degradation was proposed to occur via interactions with its scaffold binding partner Homer-3 (Tu et al., 1998; Xiao et al., 1998). In a yeast 2 hybrid screen, Homer-3 was found to interact with the S8 ATPase, a protein that forms the 19S base of the proteasome (Rezvani et al., 2012). Both Homer-3 and mGluR1α were found to coexist with S8 ATPase in differentiated HC2S2 rat ER fractions and mouse PFC fractions, and could further bind to 26S proteasomes. Overexpression of Homer-3 in differentiated PC12 cells decreased mGluR1α levels and increased its ubiquitination whereas removal of Homer-3 had the opposite effect. These findings suggested that Homer-3 was necessary to shuttle mGluR1α for proteasome degradation (Rezvani et al., 2012). It would be interesting to determine the types of chains that are formed on mGluR1α by Homer-3 manipulations since the prior study described nonproteolytic K63- mGluR1 linkages (Gulia et al., 2017). Given these interactions were identified in ER fractions, this pathway may be more relevant to the unfolded protein response (UPR) and ERAD to provide protein quality control in the ER (Hwang and Qi, 2018) (Fig. 4). Of note, Siah1A has also been recognized as a participant in the UPR pathway (Scortegagna et al., 2014).

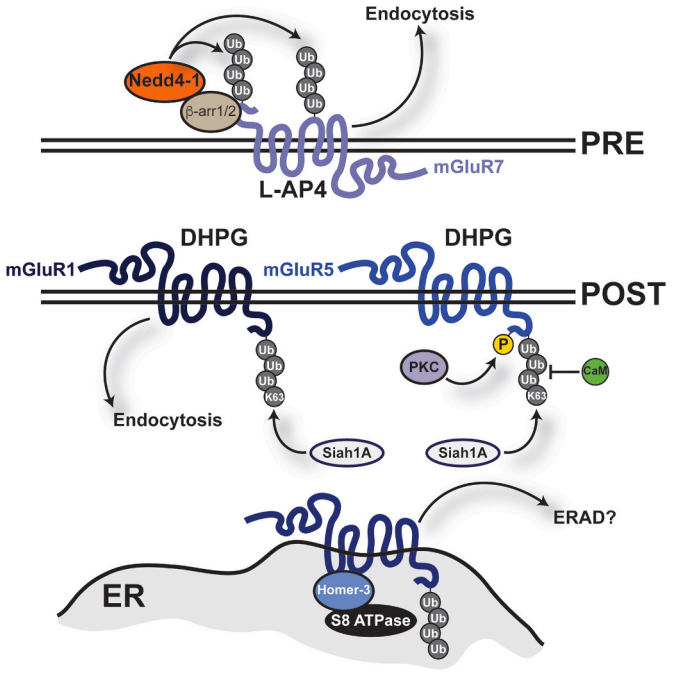

Fig. 4. Ubiquitination of mGluR receptor subunits. Top, In the presynaptic terminal (PRE), the group III mGluR agonist L-AP4 recruits β-arrestin1 and 2 to mGluR7, which in turn recruits Nedd4-1 to promote the ubiquitination of the C-terminal tail and intracellular loop of mGluR7. This modification has been proposed to increase mGluR7 endocytosis. Bottom, In the postsynaptic terminal (POST), the group I mGluR agonist DHPG promotes Siah1A-dependent ubiquitination (Ub) of mGluR1 via K63-linkages. This also facilitates ubiquitination of mGluR5 that first requires PKC-dependent phosphorylation of the mGluR5 intracellular tail, which triggers dissociation of CaM from mGluR in order for Siah1A to bind and promote K63-linked ubiquitination. mGluR1 also associates with Homer-3 and the S8 ATPase in the endoplasmic reticulum (ER). This may be critical for degradation of mGluR1 by the ERAD pathway.

6.5.2. *Ubiquitination of group III mGluRs*

The presynaptic group III mGluR, mGluR7 was identified as being ubiquitinated by Nedd4 (Lee et al., 2019). Stimulation of cells with L-AP4, an agonist for group III mGluRs led to an increase in mGluR7 ubiquitination and promoted its interaction with Nedd4. Overexpression of Nedd4 in HEK293 cells increased mGluR7 ubiquitination that required its catalytic HECT ubiquitin domain. Depletion of Nedd4 via shRNA reduced L-AP4 induced mGluR7 ubiquitination. The ubiquitination of mGluR7 was found to require Lysine residues within its C-terminus and in one of its intracellular loops. L-AP4 increased the interaction of Nedd4 with β-arrestin 1 and 2 and mGluR7. Moreover, L-AP4 mGluR7 ubiquitination also required β-arrestin 1 and 2, as depletion of these two proteins in cortical neurons blocked L-AP4 stimulated mGluR7 ubiquitination. Finally, Nedd4 could increase mGluR7 internalization similarly to β-arrestin 1 and 2 in hippocampal neurons. These findings propose a model where mGluR7 activation leads to the recruitment of β-arrestin 1 and 2, which in turn recruit Nedd4 to promote ubiquitination and internalization of mGluR7 (Fig. 4). The functional significance of this has yet to be established.

7. New insights and future perspectives on protein ubiquitination

Here I provide a historical perspective on neuronal ubiquitination, summarizing major ubiquitin signaling pathways at glutamatergic synapses. Regrettably, there are a series of additional functions that I did not discuss thoroughly in this review that include ubiquitin effects on dendritic growth, synaptic plasticity, alterations in glutamatergic postsynaptic scaffold elements, postsynaptic ubiquitin pathways that indirectly regulate glutamate receptor trafficking, and ubiquitin functions in neurological diseases. However, I refer to a series of excellent reviews that cover these important topics (Bingol and Sheng, 2011; Hamilton and Zito, 2013; Hegde, 2017; Kumar et al., 2020; Musaus et al., 2020; Tsai, 2014; Zajicek and Yao, 2020).

In reviewing the literature, there was an emergence of common themes. First and foremost (and not surprisingly), ubiquitination is necessary for all facets of nervous system function across species. Second, different ubiquitin E3 ligases have the propensity to share common substrates. This feature may be critical for functional compensatory processes essential for survival, relevant during different stages of development or activity, or required for achieving full functional outputs. For example, Nedd4, Nedd4-2, and APC^{Cdh1} have all been found to target GluA1 for ubiquitination (Fu et al., 2011; Jewett et al., 2015; Lin et al., 2011; Schwarz et al., 2010; Widagdo et al., 2015; Zhu et al., 2017). In some cases, these E3s exert their effects upon different activity manipulations. However, it is also possible that they work in concert or a sequential series to promote the turnover and trafficking of this key AMPA receptor subunit. Experiments examining these possibilities will be critical to establish a full-fledged model. Third, while some ubiquitin enzymes have a select localization, many are broadly distributed within cells to exert specific actions. For example, APC^{Cdh1} acts in the nucleus but also in pre- and postsynaptic terminals to target its substrates (Huang and Bonni, 2016). Finally, competitive actions can be driven by ubiquitin machinery. This is highlighted by the opposing actions of TRIM9 and TRIM67 in regulating axon growth and guidance (McCormick and Gupton, 2020). Hence, it is clear that ubiquitin machinery is interactive and coordinated in a spatial and temporal manner.

Intriguingly, interactions within the ubiquitin machinery are much more complicated than we have ever imagined. For example, ubiquitin E3 ligases have recently been shown to work with other E3 ligases in large complexes to ubiquitinate substrates (Horn-Ghetko et al., 2021). In some cases, this may be a similar type of mechanism that occurs with E3s that share common substrates. Moreover, the existence of a ubiquitin E4 has been shown to coordinate with E3s to elongate ubiquitin chains (Hoppe, 2005). The presence of branched ubiquitin chains and formation of linear ubiquitin chains also lends itself to unknown mechanisms

of action in the nervous system (French et al., 2021; Zajicek and Yao, 2020). And, even more compelling is the finding that non-Lysine residues on proteins can be targeted for ubiquitination by E3 ligases like PHR (Cadwell and Coscoy, 2005; Mabbitt et al., 2020; Pao et al., 2018). These findings may require researchers to revisit ubiquitin mapping of substrates, as all substrates and experiments in the nervous system favor Lysine-dependent protein ubiquitination. The specialized ubiquitin machinery in postmitotic neurons that include brain-specific ubiquitin components (Berti et al., 2002; Tai et al., 2010; Wilkinson et al., 1989) and extracellular exposed membrane associated proteasomes (Ramachandran et al., 2018; Ramachandran and Margolis, 2017) is another major layer of the ubiquitin onion that has yet to be peeled away.

Key limitations for the future include our inability to endogenously monitor spatial and temporally confined ubiquitin processes in select cell types under native conditions. Very little work has focused on monitoring protein turnover and half-lives at individual synapses or microcompartments even though we know that plasticity takes place at the level of individual cells and synapses. The inability to identify the substrates in the nervous system that are ubiquitinated by select ubiquitin enzymatic machinery is also a major limitation. This requires increasing the sensitivity of ubiquitin detection spatially in individual cells and the development of more sophisticated methods to identify and characterize ubiquitinated substrates for the massive number of cell types housed in the nervous system. Many substrates have been identified using inductive reasoning, yeast 2-hybrid approaches, proximity labeling, or differential mass spectrometry. Nevertheless, although the past 40 years has told us much about ubiquitin signaling in regulation of nervous system function, it is only the beginning of the full extent of its actions and regulations in coordinating complex neuronal processes.

Declaration of competing interest

None.

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