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Activity-Dependent Ubiquitination of GluR1 Mediates a Distinct AMPAR Endocytosis and Sorting Pathway in Hippocampal Neurons

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by

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ABSTRACT OF THE DISSERTATION

Activity-Dependent Ubiquitination of GluR1 Mediates a Distinct AMPAR Endocytosis and Sorting Pathway in Hippocampal Neurons

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The accurate trafficking of AMPA receptors (AMPARs) to and from excitatory glutamatergic synapses in the hippocampus is a critical component of mammalian learning and memory. In conjunction, recent research suggests that dysfunction of AMPAR trafficking in the hippocampus may be an underlying mechanism of Alzheimer's disease. Previous work has shown that ubiquitination of integral membrane proteins is a common post-translational modification used to mediate endocytosis and endocytic sorting of surface proteins in eukaryotic cells. Therefore, we hypothesized that mammalian AMPARs may become ubiquitinated to regulate their synaptic stability in

neurons. Here we report that mammalian AMPARs become ubiquitinated in response to their activation. Using a mutant of GluR1 that is unable to be ubiquitinated at lysines on its carboxy-terminus, we demonstrate that these ubiquitination sites are required for internalization of surface AMPARs and their trafficking to the lysosome in response to the AMPAR agonist AMPA, but not for internalization of AMPARs in response to NMDA receptor (NMDAR) agonist NMDA. Through over-expression or RNAi-mediated knockdown, we identify that a specific E3 ligase, Nedd4-1, is necessary for this process. Finally, we show that ubiquitination of GluR1 by Nedd4-1 is much more prevalent in aged neurons. Together, these data show that ubiquitination of GluR1-containing AMPARs by Nedd4-1 mediates their endocytosis and trafficking to the lysosome. Furthermore, these results provide insight into how hippocampal neurons regulate AMPAR trafficking and degradation with high specificity in response to differing neuronal signaling cues, and suggest that changes to this pathway may occur with age.

I. Introduction

Though the human brain is a complicated network of approximately 100 billion neurons making trillions of neuronal connections, it is able to receive, process, and relay information from other parts of our bodies and our environment in tenths of seconds [1]. Furthermore, networks of these cells are able to store and recall information on timescales ranging from minutes to years. The ability of neurons to mediate these complicated processes is critically dependent on their ability to maintain the structural and functional integrity of their synapses, which are the points of contact between neurons where chemical and electrical signals are transmitted. Most signaling between neurons in the brain occurs at chemical synapses, where neurotransmitter released from the pre-synaptic compartment on the axon of one neuron travels across the synaptic cleft and binds to corresponding ion channel receptors located on the surface of the post-synaptic dendritic region of another neuron. The binding of neurotransmitter causes these post-synaptic channels to open, allowing ions such as Na^+, K^+, or Ca^{2+} to flow into the neuron, thereby changing the electrical charge difference, or membrane potential, across the neuron's plasma membrane. This depolarization, called an action potential, is the basis of electrical signaling between neurons in the brain.

The Hippocampus

An area of the brain where synaptic signaling is very well studied is the hippocampus. In part, this is due to its distinct architecture, which has allowed scientists to more easily characterize its neuroanatomical and electrophysiological properties than

other brain regions. Furthermore, it is now well known that the hippocampus is a crucial brain region for learning and memory storage. This finding can initially be credited to studies on the patient H.M. in the 1950s. Suffering from epilepsy, H.M. underwent bilateral hippocampal removal. Afterwards, though most brain function was unaffected, and both his memories from before the surgery as well his short term memory were intact, H.M. was unable to retain any new memories [2]. Therefore, it seemed that removal of the hippocampus profoundly affected H.M's ability to transfer short-term learning and memory to long-term memory. Since the landmark studies of H.M, many studies have focused on uncovering the molecular mechanisms underlying memory and learning in the brain through studies of hippocampal neurons.

AMPA Receptors

Changes in synaptic strength, known as synaptic plasticity, are believed to be the basis of learning and memory. Specifically, these modifications occur at excitatory, glutamatergic synapses in the hippocampus, and involve either changes in neurotransmitter release at the pre-synaptic side of the synapse or in neurotransmitter receptor number or function on the post-synaptic side. There are three major classes of glutamatergic receptors at excitatory synapses: ionotropic receptors, metabotropic receptors, and kainate receptors. Of these classes of receptors, the ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), along with N-methyl D-aspartate receptors (NMDARs), have been found to be crucial for synaptic plasticity related to learning and memory in the hippocampus (Figure 1-1). In particular,

AMPARs are integral in regulating changes in synaptic strength due to their rapid trafficking to and from the synapse and their fast response to neurotransmitter release.

AMPARs are comprised of combinations of four highly similar subunits, GluR1-GluR4 [3]. The subunit composition of AMPARs varies depending on brain region and neuronal maturity. Differences in subunit composition contribute to a variance in the functional properties of AMPARs [4]. In mature hippocampal synapses, most AMPARs are comprised of either GluR1/GluR2 or GluR2/GluR3 subunits, along with a small population of GluR1 only receptors. The extracellular (N-terminal) and transmembrane regions of all four AMPAR subunits are highly similar, but they vary in the length and composition of their intracellular (C-terminal) cytoplasmic tails. GluR1 and GluR4 have long intracellular tails, while GluR2 and GluR3's tails are short. Additionally, GluR2 undergoes RNA editing so that a specific glutamine codon in the pore of the ion channel is replaced by arginine. This change lowers GluR2-containing AMPARs' channel kinetics, calcium permeability, and neurotransmitter affinity [5]. Meanwhile, AMPARs lacking GluR2 subunits have a high permeability for calcium and channel conductance, but undergo a voltage-dependent block at positive membrane potentials due to interactions of intracellular polyamines with the channel pore [6]. Therefore, the inclusion of the GluR2 subunit can have large effects on the properties of synaptic AMPARs, and consequently synaptic transmission, in neurons.

These differences in subunit composition also regulate when and how AMPARs are recruited to synapses. GluR1 and GluR4-containing AMPARs are inserted into the synapse at a higher rate upon activation of NMDARs or other increases in neuronal

activity that may stimulate synaptic plasticity [7]. Meanwhile, GluR2/GluR3 AMPARs are rapidly cycled in and out of synapses under basal conditions, regardless of changes in synaptic strength [8, 9].

These differences in trafficking are due, in large part, to discrete differences in the tail composition of the different AMPAR subunits that allow them to differently interact with other synaptic proteins to regulate their recruitment and stability at the synapse. The C-terminal tail of GluR2 has been shown to bind to *N*-ethylmaleimide-sensitive fusion protein (NSF), which seems to mediate insertion and stabilization of surface GluR2 at synaptic sites [10]. The tail of GluR2 also interacts with the synaptic protein PICK1, which mediates AMPAR endocytosis and recycling, and also may assist in GluR2 ER exit [11]. GluR2 and GluR3 both interact with GRIP1, which appears to play a role in the stabilization and removal of AMPARs from the synapse [12]. Meanwhile, the GluR1 intercellular tail has been shown to interact with the synaptic protein SAP97 [13, 14]. This interaction is necessary for proper localization of GluR1 to synapses, though the interaction between the two proteins may occur in the ER [15]. GluR1 has been shown to interact with the cytoskeletal protein 4.1N in a palmitoylation-dependent manner that inhibits its endocytosis from the plasma membrane [16]. Finally, AMPARs have been shown to directly interact with TARP family members, including the protein Stargazin, to mediate their trafficking in the secretory pathway and delivery to the plasma membrane [17]. This interaction has also been shown to modify the functionality of the AMPARs [18, 19].

Furthermore, post-translational modifications, specifically phosphorylation, on amino acids of the C-terminal tails of GluR1 and GluR2 have been shown to mediate their insertion and removal from the synapse. Phosphorylation of GluR1 at serine residue (Ser) 831 occurs during induction of long-term potentiation (LTP) in hippocampal neurons, via the kinase CaMKII [20, 21]. It is thought that Ser831 phosphorylation serves as a signal to send GluR1-containing AMPARs to the synapse in order to increase synaptic strength. Phosphorylation of GluR1 at Ser845 by the kinase PKA seems to play several crucial roles in LTP. It has been shown that phosphorylation at this site affects the open-channel probability of GluR1-containing AMPARs [22]. Ser845 phosphorylation also recruits GluR1-containing AMPARs to extra-synaptic sites, and prepares them for lateral diffusion into the synapse [23, 24]. Furthermore, it has been shown that phosphorylation of Ser845 allows GluR1-containing AMPARs to enter a recycling pathway after endocytosis from the plasma membrane, leading to their re-insertion in the synapse [25]. Likewise, dephosphorylation at these sites (Ser831 and Ser845) occurs during hippocampal long-term depression [26].

Ubiquitin-Proteasome System

Another post-translational modification shown to mediate the stability and trafficking of cellular proteins is ubiquitination. The ubiquitin-proteasome system (UPS) is a highly regulated proteolytic pathway that mediates a majority of protein degradation in eukaryotic cells [27]. The discoverers of this pathway, Rose, Hershko and Ciechanover, were awarded the Nobel Prize for Chemistry in 2004. The UPS is comprised of three classes of enzymes, which work together to attach single or chains of

ubiquitin molecules to lysine resides of target proteins. Ubiquitin, a 76 amino acid protein, is first activated by the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin ligase (E3) attaches the ubiquitin to a lysine residue of the specified substrate. While there are only 1-2 E1s, and only a small number of E2s in cells, there are at least hundreds of E3 ligases [28]. This is because E3 ligases have a high level of specificity for their target substrates. Each E3 pairs up with one or more E2 to recognize a set of substrates that share one or more signals for ubiquitination. These ubiquitination signals can range from changes in a protein's phosphorylation status, specific amino acid sequences recognized as binding domains by a ligase, or conformational changes to a protein or its binding partners that suddenly allow access for ubiquitination by the ligase [29].

Diversity of Ubiquitination

The UPS is best studied for its role in protein degradation. It is well established that proteins with ubiquitin chains attached are recognized by the 26S proteasome and are degraded. However, many studies have shown that ubiquitination can sometimes mediate the trafficking of cellular proteins without leading to their degradation by the proteasome (Figure 1-2) [30]. A major factor in determining the fate of ubiquitinated proteins is the number of ubiquitin attached to the substrate, and the structure of these ubiquitin chains. Specifically, proteins can undergo three types of ubiquitination: mono-ubiquitination (the attachment of a single ubiquitin to a protein), multi-mono-ubiquitination (the attachment of single ubiquitins to multiple lysine residues of a

protein), and poly-ubiquitination (the attachment of ubiquitin chains to a protein). While poly-ubiquitination is mainly associated with proteasome-mediated degradation, mono- and multi-mono-ubiquitination have been shown to mediate endocytosis and endocytic sorting of proteins through vesicles in the secretory/endocytic pathway [31]. One hypothesis for why ubiquitinated proteins can have different fates is that the structure of the attached ubiquitin chains can differ. Since ubiquitin itself contains seven internal lysine residues, ubiquitin chains can undergo seven different linkage structures (K6, K11, K27, K29, K33, K48, and K63). While the specificity and function of some of these linkages is still unknown, it has been shown that K48 linkage mainly targets proteins to the proteasome, while K63 linkage may be involved in DNA damage tolerance, the endocytic pathway, and ribosomal protein synthesis [32]. Also, it is thought that the length of ubiquitin chains can regulate a protein's degradation [33]. Specifically, it has been shown that the proteasome recognizes ubiquitin chains greater than four molecules in length [34]. Likewise, research suggests that attachment of 1-3 ubiquitin molecules tends to regulate protein trafficking, such as endocytosis of plasma membrane proteins or the endocytic sorting of proteins, or protein function. Cells also contain molecules called deubiquitinating enzymes (DUBs), which can rapidly remove ubiquitin molecules from target proteins, thereby modifying their cellular fate. Finally, a large unanswered question in the field is how E3 ligases decide to mono- or poly-ubiquitinate their target proteins. In the case of the protein p53, the ubiquitin ligase Mdm2 is recruited under conditions where p53 undergoes mono-ubiquitination, but the ligase p300 is recruited when p53 becomes poly-ubiquitinated [35, 36]. Also, it's been shown that a specific ligase can differentially ubiquitinate a target protein based on several factors, such as the

structure or cellular localization of the protein, or the protein's interactions with other proteins at the time of ubiquitination. For instance, the ligase Cbl has been shown to poly-ubiquitinate several cytoplasmic proteins, such as Src and Abl tyrosine kinases, leading to their proteasomal degradation, while mono-ubiquitinating plasma membrane proteins such as receptor tyrosine kinases (RTKs) for their internalization [37-39].

Ubiquitin-mediated Endocytosis

Though ubiquitin-mediated endocytosis has been shown to occur in many eukaryotic cell types, it was first discovered in yeast, and has been most extensively studied in that system, where it seems a majority of plasma-membrane proteins undergo ubiquitin-mediated endocytosis. The first indication that ubiquitination was required to internalize surface proteins in yeast was through studies of the yeast peptide transporter Ste6p. Researchers observed that in yeast with endocytosis impairments, there was an accumulation of ubiquitinated Ste6p in plasma membrane fractions. However, this accumulation could be slowed if ubiquitin-conjugating machinery in the cell was also mutated [40]. Further studies on the yeast α–factor receptor, Ste2p, which binds the α–factor mating pheromone, provided further insight into the mechanisms of ubiquitin-mediated endocytosis. Ste2p is a G-protein-coupled receptor with seven transmembrane sections, an extra-cellular amino terminus, and a long cytoplasmic tail. In the absence of ligand, Ste2p undergoes slow, constitutive internalization and trafficking to the vacuole for degradation. In the presence of α–factor, the rate of Ste2p internalization increases 10 fold, and its carboxy-terminus undergoes phosphorylation and ubiquitination [31]. In cells with mutated ubiquitin machinery, Ste2p was no longer internalized in response to

α–factor [41]. Also, if lysine residues on the tail of Ste2p are changed to arginine, blocking ubiquitination at those sites, the endocytosis rate of Ste2p was severely inhibited. Interestingly, changing single lysine residues on the tail did not have this effect, suggesting more than one lysine may be utilized to induce ubiquitin-mediated endocytosis of proteins. Mutating specific serine phosphorylation sites on the tail also inhibited its endocytosis, suggesting that ubiquitination and phosphorylation signals may work in conjunction to mediate protein internalization. Together, these results were the first to indicate that lysine residues on the intracellular tail of a plasma membrane protein could regulate its endocytosis in an ubiquitin-mediated manner in eukaryotic cells.

Since this initial work in yeast, it has become clear that many plasma membrane proteins in mammalian cells also undergo ubiquitin-mediated endocytosis. The first mammalian receptors shown to be ubiquitinated were the platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) [42, 43]. These receptors are tyrosine kinase receptors that stimulate a signal transduction cascade when activated. Binding of ligand to these receptors stimulates their internalization and degradation via ubiquitination of their cytoplasmic tails. Recently, another class of mammalian receptors from the G-protein coupled receptor family has been shown to be ubiquitinated. Specifically, the β2-adrenergic receptor and the chemokine receptor CXCR4 undergo ubiquitination when stimulated. Interestingly, for these receptors, it was shown that ubiquitination was not required for their internalization, but was required for proper endosomal sorting and trafficking to the lysosome [44, 45]. Several mammalian transport proteins have also been shown to be ubiquitinated. The first to be discovered

was the amiloride-sensitive epithelieal sodium channel (ENaC). ENaC regulates salt reabsorption in the distal colan and lung epithelia, and elevation of its activity leads to hypertension. Deletions in the cytoplasmic tail of one of the ENaC subunits was found to result in a hereditary form of hypertension, called Liddle's syndrome [46, 47]. The C-terminal tail of ENaC has been shown to interact with the E3 ligase Nedd4-1, while specific lysine residues on several ENaC subunits are targeted for ubiquitination [48]. Similarly, the presence of dopamine transporter (DAT) at the plasma membrane of dopaminergic neurons has been shown to be mediated by ubiquitination. DAT undergoes constitutive ubiquitination, which can be significantly increased upon protein kinase C (PKC) activation [49]. This ubiquitination was found to be K63-linkage mediated, and each ubiquitin chain attached to DAT contained between 2-3 ubiquitin molecules. Ubiquitination of DAT occurs at the plasma membrane, but is thought to be necessary for proper DAT trafficking to the lysosome for degradation. The similarities between mechanisms of ubiquitin-mediated endocytosis in yeast, where most surface proteins undergo ubiquitin-mediated endocytosis, and recent discoveries of this process in mammalian cells, suggest that ubiquitin-mediated endocytosis and endocytic sorting may also play a prominent role in the trafficking of surface proteins in higher order systems.

The UPS and Neurological Diseases

While the UPS plays is important for regulating protein populations in all eukaryotic cells, it has recently become an area of intense focus in the neurobiology field, as emerging work has indicated that the UPS plays a central role in neuronal function. Interest in the UPS from a neurological standpoint began with the observation that

protein aggregates from several neurological disorders, such as Alzheimer's disease and Parkinson's disease, contained large amounts of ubiquitin. Together, these results suggested that dysfunction of the UPS pathway may be an underlying cause of these neurodegenerative diseases [50]. More recently, several specific molecules of the UPS pathway have been shown to play central roles in distinct neurological disorders. For instance, mutations in the gene UBE3A, which encodes for the HECT domain E3 ligase Ube3a, have been linked to the both autism and Angelman syndrome, disorders resulting from defects in neuronal development [51, 52]. Ube3a has been shown to localize in dendrites and spines, and loss of Ube3a causes decreases in spine number and length [53]. Also, recent work has shown that model mice deficient in Ube3a had large impairments in experience-dependent development of cortical synapses and loss of plasticity in neocortical circuits [54].

Furthermore, the DUB UCH-L1 has been implicated in several neurodegenerative diseases. Specifically, the *gracile axonal dystrophy (Gad)* mutant mouse suffers from developmental sensory and adult motor ataxia, caused by axonal loss over time. These mice have a deletion in the UCH-L1 locus [55]. Also, in a case study of familial Parkinson's disease (PD), it was found that family members suffering from an autosomal dominant form of PD carried a mutation in the UCH-L1 locus that significantly reduced the deubiquitinating activity of UCH-L1 protein [56]. An E3 ligase called Parkin has also been linked PD. Genetic studies have shown that a variety of mutations to the Parkin gene PARK2 produce PD phenotypes in patients [57]. Parkin has been found to target many proteins important for synaptic function, such as α-synuclein,

synphillin, synaptotagmin XI, Eps15, and PICK1 [58-60]. Over-expression of Parkin in hippocampal neurons reduces synaptic transmission, while knockdown of Parkin enhanced transmission and induced an increase in glutamatergic synapses, leading to excitotoxicity in those neurons [61].

While studies of the UPS in neurons originally focused on the pathway's role in neurological diseases, it has since become clear that the UPS plays an important role in normal neuronal function. Though too numerous to describe in detail in this thesis, the UPS pathway has been implicated in a wide array of neuronal processes, including axon growth and guidance, synaptogenesis, and pre- and post-synaptic function by mediating the turnover of many synaptic proteins [62-64].

In this thesis, I describe two major findings that provide a new model for how mammalian AMPARs are removed from the plasma membrane in response to direct activation and properly sorted into endocytic pathways that differentially lead to their recycling or degradation by the lysosome. In Chapter 2, I establish that the AMPAR GluR1 subunit undergoes rapid ubiquitination that is dependent on the direct activation of AMPARs with the pharmacological agonist AMPA and a supply of extracellular calcium. I demonstrate that this ubiquitination occurs at C-terminal lysine residues of GluR1, and is necessary for endocytosis in response to AMPA, but not for endocytosis induced by the NMDAR agonist NMDA. In Chapter 3, I describe the discovery of an E3 ligase, Nedd4-1, that directly interacts with and ubiquitinates GluR1 to stimulate its internalization. Using over-expression and RNAi to alter Nedd4-1 expression levels in neurons, I demonstrate that Nedd4-1 is necessary for AMPA-stimulated endocytosis of GluR1 and

its trafficking to the lysosome. Together, these findings provide evidence for a previously unknown pathway of AMPAR trafficking mediated by ubiquitination and identifies multiple mechanisms for how this pathway is regulated.

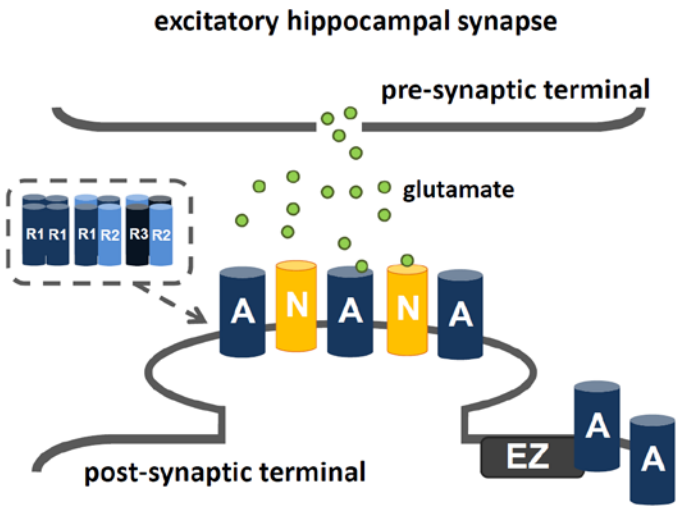

Figure 1-1 Hippocampal excitatory synapse

A majority of synapses in the hippocampus are excitatory synapses, meaning that when activated, they produce a depolarizing response in the neuron. At these synapses, the neurotransmitter glutamate is released from the pre-synaptic terminal, where it travels across the synaptic cleft and binds to post-synaptic AMPA (A) and NMDA (N) receptors. AMPA receptors are comprised of four subunits (GluR1-GluR4), which together form a functional channel. Binding of glutamate causes these channels to open, allowing Na+ and Ca2+ to flow into the neuron, causing it to depolarize. AMPA receptors can also reside on the surface of neurons in extra-synaptic locations called endocytic zones (EZ), where they are not exposed to glutamate, but can laterally diffuse into the synapse when necessary.

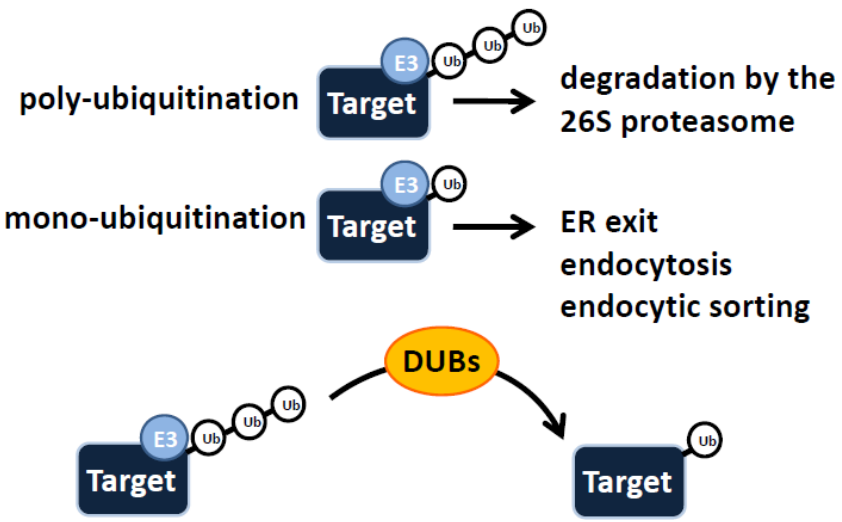

Figure 1-2 Diversity of ubiquitination and function

Diversity in the length and structure of ubiquitin chains attached to substrates can dictate their fate in cells. When proteins are poly-ubiquitinated via the attachment of ubiquitin chains to lysine residues within the protein, this most often leads to its degradation by the 26S proteasome. If proteins are mono-ubiquitinated, this often mediates the trafficking of the protein, but does not lead to its degradation by the proteasome. Mono-ubiquitination has been shown to be involved in removal of proteins from the ER and the plasma membrane, and mediating their sorting through endocytic pathways. Ubiquitination can also be reversed by de-ubiquitinating enzymes (DUBS), which can either completely remove ubiquitin from substrates or can remove ubiquitin from poly-ubiquitin chains, trimming them to shorter lengths that may no longer be recognized by the proteasome.

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II. AMPA Receptors Undergo Activity-Mediated Internalization and Endocytic Sorting in Neurons

Introduction

Activation of AMPARs has been shown to induce their endocytosis and subsequent trafficking into endosomal/lysosomal sorting pathways in hippocampal neurons [1-3]. Since ubiquitination is a well-studied post-translational modification that mediates endocytosis and endocytic sorting of surface receptors, we asked if activation of AMPARs induced their ubiquitination.

Results

To examine this, we treated dissociated neuronal cultures (DIV>14) with AMPA (100μM, 10min.), an AMPAR agonist, and immunoprecipitated the resulting lysates with anti-GluR1 antibodies. The IPs were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-ubiquitin antibodies. We found that AMPA caused GluR1 to be ubiquitinated (5.0±0.60 for AMPA to 1±0.23 for control)(Figure 2-1 A,B). Significant increases in ubiquitination were not observed for the AMPAR GluR2 subunit (2.1±0.49 for AMPA to 1±0.40 for control) or the N-methyl D-aspartate receptor (NMDAR) NR1 subunit (1.2±0.15 for AMPA to 1±0.21 for control), suggesting AMPA-induced ubiquitination occurs primarily on the GluR1 subunit. To ensure preferential enrichment of GluR1 in the IPs, a buffer containing high amounts of detergents (1% TX-100, .2% SDS) was used to aid the dissociation of AMPAR tetramers and other associated post-synaptic density (PSD) proteins. Quantification of the amount of GluR1

or GluR2 in the GluR1 and GluR2 specific IPs showed enrichment for those specific subunits (1±0.07 of GluR1 to 0.6±0.04 GluR2 in GluR1 IPs; 1±0.07 GluR2 to 0.4±0.05 GluR1 in GluR2 IPs)(Figure 2-1 C). Also, a silver stain of the GluR1 IP showed isolation of a single band which migrated at the predicted size of GluR1 (~117kD), with only moderate amounts of another unidentified protein visible (~80kD) plus antibody heavy chain (~55kD)(Figure 2-2 A). On lower exposures of the Western blot probed with anti-GluR1 antibody, the majority of immunoprecipitated GluR1 appears to be unmodified based on size. However, upon higher exposures, high molecular GluR1 reactivity is visible at the size predicted for ubiquitinated GluR1 in AMPA-treated IPs, but absent from untreated IPs (Figure 2-2 A). The ubiquitin immunoreactivity was specific, as exposing the ubiquitin antibody to ubiquitin-bound agarose prior to probing the Western blot resulted in a loss of ubiquitin immunoreactivity (Figure 2-2 B). Together, these results suggest that the ubiquitination visible by Western blot after application of AMPA occurs on the GluR1 subunit. After AMPA stimulation, ubiquitinated GluR1 consistently appeared as two distinct bands slightly larger in size than unmodified GluR1 on Western blots. This suggests GluR1 is ubiquitinated by single ubiquitin molecules or short ubiquitin chains which cause a modest shift in GluR1 molecular weight. To estimate the molecular weight size of this population of GluR1, we plotted the electrophoretic mobilities of the molecular weight standards from each Western blot against their known molecular weights as previously described (Figure 2-2 C-E) [1]. After deriving a logarithmic trend line through these points, we used the resulting trend line equation to calculate the predicted size of the two main ubiquitinated GluR1 bands based on their electrophoretic mobility (Figure 2-2 D). The estimated size

of these bands was found to be highly similar over multiple experiments. The ubiquitinated GluR1 band immediately above unmodified GluR1, called GluR1-Ub3, was estimated to be 24 kD larger than GluR1 (Figure 2-2 E). As ubiquitin is approximately 8 kD, we hypothesize that this band represents GluR1 with three ubiquitin molecules attached. A slightly larger ubiquitinated GluR1 band, called GluR1-Ub4, was estimated to be 33 kD larger than unmodified GluR1, suggestive of GluR1 with four ubiquitin molecules attached. These ubiquitin molecules could be attached to distinct lysine residues of GluR1 or combined to form short ubiquitin chains. Together, these findings indicate that application of AMPA induces the ubiquitination of the GluR1 subunit of AMPARs.

It was previously reported that in response to AMPA, AMPARs were endocytosed and targeted to the lysosome for degradation [2]. However, since ubiquitination of certain proteins can mediate their degradation by the proteasome, we confirmed that application of AMPA caused AMPAR degradation by the lysosome and not the proteasome. Total GluR1 in neuronal cultures decreased with increased exposure to AMPA (100μM, 5-60 min.)(Figure 2-3 A,B). This loss was not blocked by co-application of the proteasomal inhibitor MG-132 (25μM) but was blocked by the lysosome inhibitor leupeptin (200μg/ml). Therefore, application of AMPA induces the ubiquitination and endocytosis of GluR1, as well as its trafficking to the lysosome for degradation.

Ubiquitination of AMPARs is Dependent on AMPAR Activation and Calcium

Previous work has suggested that the relative activation of AMPARs by direct or indirect stimuli differentially manipulates their endocytosis and endocytic sorting [2-4]. To determine if GluR1 ubiquitination was specific to AMPAR activation, we treated neuronal cultures with AMPA in the presence of the AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione(CNQX, 40µM, 30 min.). We found that CNQX significantly attenuated GluR1 ubiquitination (4.5±0.61 for AMPA and 2.5±0.53 for CNQX+AMPA)(Figure 2-4 A,B). In contrast, blocking NMDARs with the NMDAR antagonist DL-2-Amino-5-phosphonopentanoic acid (APV, 50µM, 30 min.) had no effect on AMPA-induced ubiquitination of GluR1 (4.5±0.61 for AMPA and 5.0±0.89 for APV+AMPA). Furthermore, stimulating NMDARs directly with the agonist NMDA (25µM, 10 min.) did not induce GluR1 ubiquitination (1.7±0.51 for NMDA). This indicates NMDAR activation is not sufficient for AMPAR ubiquitination. Finally, removing calcium, a key regulator of many synaptic signaling pathways, from the cell media prior to application of AMPA completely abolished GluR1 ubiquitination (4.5±0.61 for AMPA to 1.5±0.40 for Ca^{2+}-free AMPA). These results indicate that the rapid ubiquitination of GluR1 in response to AMPA is dependent on the direct activation of AMPARs and requires external calcium entry into the neurons, most likely through voltage-gated calcium channels or sources other than NMDARs.

GluR1 Phosphorylation at Serine 845 Affects its Ubiquitination

It has been reported that de-phosphorylation of GluR1 at serine 845 on its C-terminal tail occurs during endocytosis [5-7]. Also, in many eukaryotic cell types, changes in phosphorylation status can serve as a recruitment signal for ubiquitination-

mediated endocytosis of proteins [8]. Therefore, we examined whether blocking phosphorylation of GluR1 at serine 845 would affect its ubiquitination. We created a phosphorylation mutant of GFP-tagged GluR1 where serine 845 was changed to alanine (GluR1-S845A). We co-transfected GluR1-S845A or GluR1-WT into HEK293T cells with HA-tagged ubiquitin, isolated GluR1 by immunoprecipitation, and assessed its ubiquitination status via Western blot. We observed increased ubiquitination on GluR1-S845A, indicating that loss of phosphorylation at serine 845 increases ubiquitination of GluR1 (Figure 2-5 A). We next performed experiments to address this phenomenon in neurons. Previous research has shown that GluR1 is phosphorylated at serine 845 by the kinase PKA [5]. Therefore, we hypothesized that blocking phosphorylation at this site through inhibition of PKA may increase ubiquitination of GluR1. Dissociated neuronal cultures were treated with H-89 (2 μM, 4 hours), a PKA antagonist, and the resulting lysates were immunoprecipitated with GluR1 antibodies. We found that blocking PKA activity increased GluR1 ubiquitination (Figure 2-5 B).

Ubiquitination of GluR1 C-terminal Lysines Regulates Their Surface Accumulation and Internalization

Surface GluR1 contains three intracellular transmembrane loops and a C-terminal tail that are exposed to ubiquitination machinery inside the cell. Of these domains, only the C-tail contains lysine residues (at amino acids 813, 819, 822, and 868) that could serve as sites for ubiquitin attachment. This region of mammalian GluR1 shares homology to a region in *C. elegans* GLR-1 that has been shown to be ubiquitinated, and also to a region of mammalian GluR2 that contains an endocytosis signal (Figure 2-6 A,

bottom panel) [3, 9]. Since AMPA-induced GluR1 ubiquitination consistently appears as multiple distinct bands (Figures 2-1, 2-2, 2-4), and the size of these bands is suggestive of GluR1 with three to four ubiquitins attached (Figure 2-2 E), we hypothesized that GluR1 may be ubiquitinated at multiple sites. To explore this, we mutated the C-terminal tail lysine residues of GFP-tagged GluR1 (GluR1-WT) to arginine so they could no longer be ubiquitinated, creating GluR1-4KR (Figure 2-6 A, top panel).

We next asked if the loss of C-terminal ubiquitination sites would alter surface expression levels of GluR1. To do this, we expressed GFP-GluR1-WT or GluR1-4KR in mature hippocampal neurons using Sindbis virus as previously reported [10]. We limited Sindbis virus expression to ~18-22 hours to maintain cell viability. After infection, neurons were live-labeled with anti-GFP Alexa594 fluorophore to visualize surface GluR1, permeabilized, and labeled with anti-GFP Alexa488 fluorophore to visualize internal GluR1 before imaging by confocal microscopy. We observed a significant increase in the intensity of surface GluR1-4KR compared to GluR1-WT in both the soma and dendrites of infected neurons (1± 0.06 for GluR1-WT to 1.5± 0.07 for GluR1-4KR in soma, 1± 0.03 for GluR1-WT to 1.2± 0.03 for GluR1-4KR in dendrites, *$p<0.05$, unpaired Student's t-test)(Figure 2-6 B,C). We determined this result was not due to increased insertion of newly synthesized proteins in the membrane by treating infected neurons with Brefeldin A (BFA). BFA is an antibiotic that inhibits intracellular protein transport by disrupting trafficking from the trans-Golgi complex. After BFA treatment (5µg/ml, 45min.), we observed that surface GluR1-4KR immunofluorescence was much more stable than GluR1-WT (1±0.08 for GluR1-WT to 0.5±0.07 for GluR1-WT+BFA;

1±0.07 for GluR1-4KR to 0.8±0.10 for GluR1-4KR+BFA)(Figure 2-7). These results are consistent with GluR1-4KR having increased surface levels due to decreased internalization and lysosomal degradation.

To explore if GluR1-4KR undergoes normal activity-mediated endocytosis, mature hippocampal neurons were infected with Sindbis GFP-tagged GluR1-WT or GluR1-4KR, live-labeled with anti-GFP antibodies, and exposed to AMPA (100μM) or NMDA (25μM) for 10 min at 37°C. Cells were then fixed and exposed to unlabeled secondary antibodies to block any GFP antibody remaining on the cell surface before permeabilizing. Then, internalized pools of GluR1-WT or GluR1-4KR were labeled with Alexa568-conjugated secondary antibody. As expected, both AMPA and NMDA significantly induced endocytosis of surface GluR1-WT in the soma and dendrites of infected neurons compared to control-untreated neurons (1±0.14 for control to 1.9±0.29 for AMPA and 1.7±0.30 for NMDA in soma; 1±0.11 for control to 1.4±0.16 for AMPA and 1.4±0.15 for NMDA in dendrites)(Figure 2-8 A,B). In contrast, AMPA-induced endocytosis was completely abolished for GluR1-4KR (1±0.14 for control to 0.8±0.21 for AMPA in soma; 1±0.11 for control to 0.8±0.14 in dendrites). However, GluR1-4KR was still significantly internalized in response to NMDA (1±0.14 for control to 1.6±0.22 for NMDA in soma; 1±0.11 for control to 1.3±0.10 in dendrites). Since NMDA-induced GluR1-4KR endocytosis was similar to GluR1-WT, this suggests that changing the C-terminal tail lysines to arginines did not affect GluR1's functionality or ability to interact with proteins that assist in its internalization. These results indicate that ubiquitination at

C-terminal lysines is needed for AMPA- but not NMDA-induced internalization of GluR1.

Conclusion

Together, these experiments indicate for the first time that mammalian AMPARs undergo ubiquitination. This ubiquitination is dependent on direct activation of the AMPARs and calcium influx into hippocampal neurons, but is independent of NMDAR activation or signaling. AMPAR ubiquitination occurs specifically at C-terminal lysine residues of the GluR1 subunit. Ubiquitination at these sites is necessary for mediating internalization of AMPARs from the plasma membrane of neurons and mediating their trafficking to the lysosome for degradation, but is not required for other AMPAR internalization pathways that may lead to their recycling back to the plasma membrane. The identification of this novel post-translational modification to mediate AMPAR trafficking should be of great interest to the field as the regulation of AMPAR removal from the synapse is a crucial component of learning and memory in the hippocampus. Future studies to identify the involvement of other molecules, such as E3 ligase and clathrin machinery, or the signaling mechanisms mediating recruitment of ubiquitination, are currently planned. Also, since mutations to the UPS pathway are a well known component of Alzheimer's disease, where memory loss is also a central component, the discovery of AMPAR ubiquitination may provide insight into molecular mechanisms underlying that disease.

Materials and Methods

Antibodies and Reagents: Antibodies were obtained as follows: pAb GluR1, pAb GluR2 (Millipore), pAb surface (N-terminal) GluR1 (Calbiochem), mAb ubiquitin (P4D1) and pAb GFP (Santa Cruz), anti-GFP secondary antibody (Invitrogen), pAb Actin (Cytoskeleton Inc.), AMPA, NMDA, CNQX, APV, and H-89 (Tocris), BFA (Invitrogen), N-ethylmaleimide (NEM) and leupeptin (Sigma), MG-132 (Biomol).

Hippocampal Cultures: Rat dissociated hippocampal or hippocampal and cortical cultures from postnatal day 1 were plated onto poly-D-lysine coated coverslips, 35 mm dishes (Mattek), or 6 well plastic dishes and maintained in B27 supplemented Neurobasal media (Invitrogen) until 14-38 days in vitro.

Immunoprecipitations: Rat hippocampal tissue or hippocampal cultures were homogenized in precipitation buffer (PB) (in mM: 150 NaCl, 10 Na_2HPO_4, 2 EDTA) with 1% TX-100 and 0.1-0.2% SDS, 25 µM MG-132, 25mM NEM, and protease inhibitors. HEK293T cells were lysed in RIPA Buffer plus 1% BSA with 25 µM MG-132, 25mM NEM, and protease inhibitors. Homogenates were cleared by centrifugation at 14,000 rpm at 4°C. For immunoprecipitations, cleared lysates were incubated with primary antibodies at 4°C O/N, after which protein A or protein A/G sepharose beads were added for an additional 2 hours (Pierce). Immunoprecipitations from neurons were repeated 3-8 times. Immunoprecipitations from HEK293T cells were repeated 2-4 times. For all immunoprecipitations from transfected cells, equal protein expression was verified in cell lysates by Western blot. For quantification of Western blots, protein band mean intensities were calculated using ImageJ. For Western blots measuring protein

ubiquitination, ubiquitin band mean intensities were divided by the mean intensities of the corresponding immunoprecipitated receptor. Ubiquitination values from treated IPs were normalized to values from control IPs. For Western blots measuring GluR1 levels, the GluR1 band mean intensity in each condition was normalized to the actin band mean intensity from the same sample.

Transfections and Infections: HEK293T cells, maintained in DMEM + 10% serum and Pen/Strep, were transfected with Lipofectamine 2000 (Invitrogen) or PEI (Polysciences) using recommended protocols. Hippocampal cultures were infected with Sindbis virion at DIV15-19 and allowed to express for 18-22 hours. Viral titer and transduction efficiency were monitored for all viruses made to ensure equal expression of constructs.

DNA Constructs: GFP-GluR1 obtained from R. Malinow (UCSD) was mobilized in pcDNA3.1(-) vector. GluR1-4KR and GluR1-S845A were created using PCR site-directed mutagenesis. All point mutations were verified by sequencing. For Sindbis viral expression, genes were cloned into the Sindbis virus vector SinRep5. Myc-NR1-1a and GFP-GluR2 was obtained from A. Ghosh (UCSD).

Surface Live-labeling and Endocytosis: Dissociated hippocampal neurons (DIV15-20) or HEK293T cells were live-labeled with anti-GFP secondary or anti-GluR1 antibodies for 15 min at 37°C, and then washed with PBS-MC (1X PBS, 1mM $MgCl_2$, 0.1mM $CaCl_2$). Cells were then fixed for 5-10 min. with 4% paraformaldehyde/4% sucrose at room temperature. Surface GluR1 receptors were then labeled with secondary Alexa antibodies (Molecular Probes) in PBS-MC containing 2% BSA. For endocytosis experiments, cells were pre-treated with TTX (2 μM) for 1 hour before surface labeling.

After surface labeling, cells were washed with PBS and replaced with conditioned media containing either vehicle or AMPA (100 µM) and APV (25µM), or NMDA (25µM) and glycine (20µM) for 10-15 min. at 37°C. Cells were then washed with PBS-MC and fixed. Surface receptors were labeled with untagged secondary antibodies in PBS-MC before the cells were permeabilized with PBS-MC, 2% BSA, and 0.2% Triton X-100 for 20 min. The internalized receptors were labeled with fluorophore-conjugated secondary antibodies. For endocytosis experiments, the fluorescence from the GluR1-N terminal GFP molecule was negligible post fixation as its fluorescence was determined to be detectable only at exposure times ten times greater than those used to detect antibody-labeled GFP.

Confocal Microscopy and Image Analysis: All images were taken with a Leica DMI6000 inverted microscope outfitted with a Yokogawa Nipkon spinning disk confocal head, an Orca ER high resolution B&W cooled CCD camera (6.45 µm/pixel at 1X), Plan Apochromat 40X/1.25na and 63X/1.4na objective, and an argon/krypton 100mW air-cooled laser for 488/568/647 nm excitations. All images were acquired in the dynamic range of 8 bit or 12 bit acquisition. Maximum projected confocal Z-stacks were analyzed with NIH ImageJ. For experiments analyzing surface or internalized GFP or GluR1 immunofluorescence, images were background subtracted and thresholded equally, and the integrated density of each puncta was measured using a modified ImageJ particle analysis macro. The average particle integrated density for each cell was normalized to cell size. This raw data value for each cell was divided by the average raw

data value of untreated control cells to obtain a normalized value. Values from the same treatments over multiple experiments were then combined and averaged.

Statistical Analysis: Two-tailed unpaired Student's *t* test with an alpha=.05 or ANOVA with Tukey's or Fisher LSD *post-hoc* analysis was used for determining statistical significance. Results >0.05 were not considered significant.

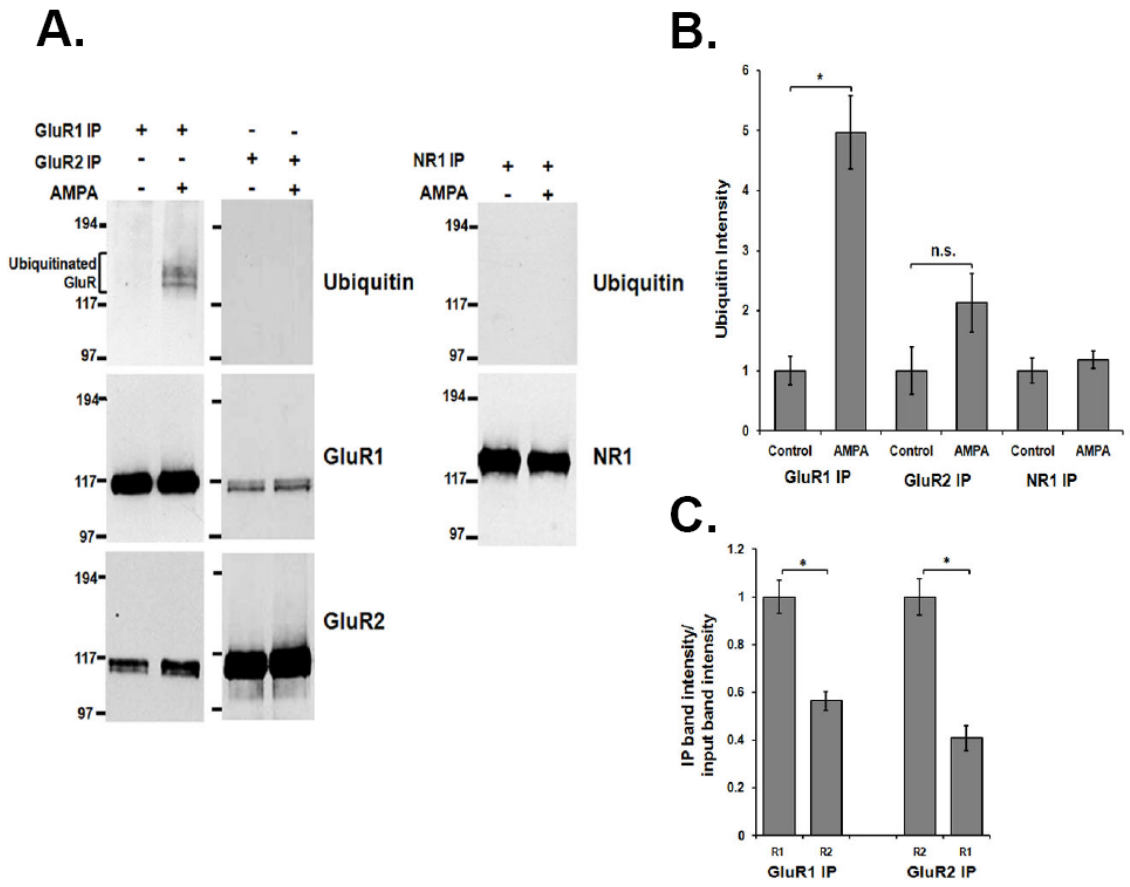

Figure 2-1 GluR1-containing AMPARs undergo activity-mediated ubiquitination

(A) Dissociated neuronal cultures were treated with AMPA (100μM, 10min.) or left untreated prior to immunoprecipitation of resulting lysates with anti-GluR1, anti-GluA2, or anti-NR1 antibodies. IPs were resolved by Western blot and probed with anti-ubiquitin antibodies and antibodies against each receptor subunit to confirm equal levels of protein in each IP. (B) Quantification of normalized mean ubiquitin intensity. (C) Quantification of IP receptor intensity divided by input receptor intensity for GluR1 and GluA2 IPs. n=3-4 IPs for each condition. *$p<0.05$, unpaired Student's t-test. Error bars=s.e.m.

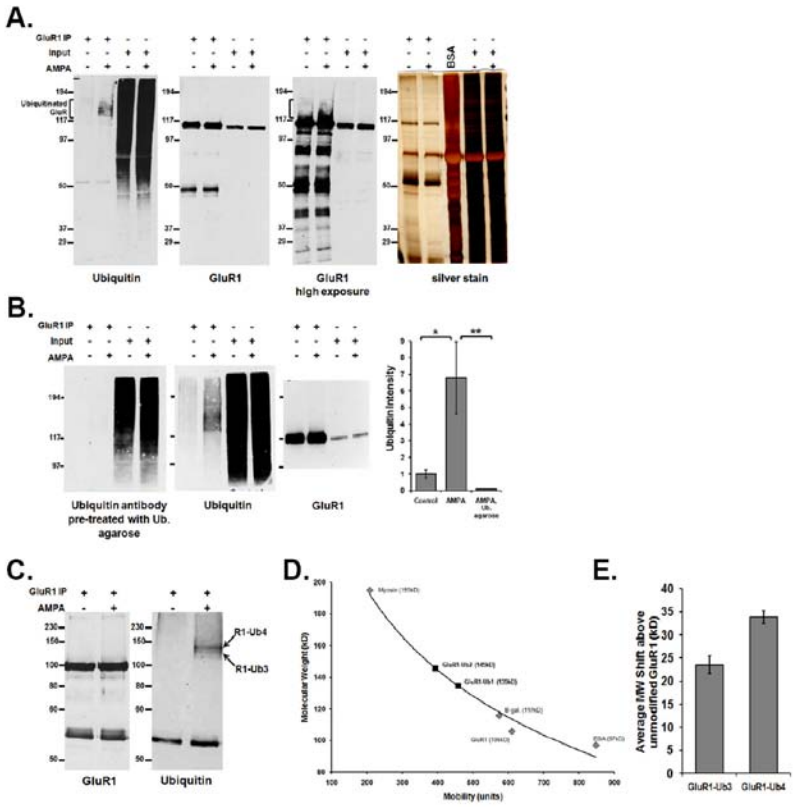

Figure 2-2 AMPARs undergo activity-mediated ubiquitination

(A) Dissociated neuronal cultures were treated with AMPA (100µM) for 10 min. or left untreated prior to immunoprecipitation with anti-GluR1 antibodies. IPs were resolved by Western blots probed with anti-ubiquitin or anti-GluR1 antibodies. IPs were also resolved on SDS-PAGE gels and silver stained to visualize all immunoprecipitated proteins. (B) Western blots from IPs prepared as in (A) were probed with solution containing anti-ubiquitin antibodies pre-absorbed with ubiquitin-bound agarose. The same Western blots were then re-probed with new anti-ubiquitin antibodies and anti-GluR1 antibodies. Quantification is of ubiquitin intensity divided by receptor intensity for each IP (1±0.24 for control, 6.8±2.18 for AMPA, 0.1±0.002 for AMPA+ubiquitin agarose). n=3 IPs for each condition. *$p<0.05$, **$p<0.01$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m. (C) An example Western blot from IPs prepared as in (A) to show molecular weight shift of ubiquitinated GluR1 species, which consistently appears as two distinct bands when labeled with anti-ubiquitin antibodies. (D) An example graph where the electrophoretic mobilities of the molecular weight standards from (C) were plotted against their known molecular weight. A best fit logarithmic trend line was generated, and the resulting formula was used to estimate the size of ubiquitinated GluR1. This calculation was repeated over 5 experiments. (E) Quantification of the size of ubiquitinated GluR1 based on (D), averaged over 5 experiments. The two ubiquitin-immunopositive bands present in the GluR1 IP after application of AMPA are approximately 24 kD (R1-Ub3) or 32 kD (R1-Ub4) larger than unmodified GluR1. Error bars=s.e.m.

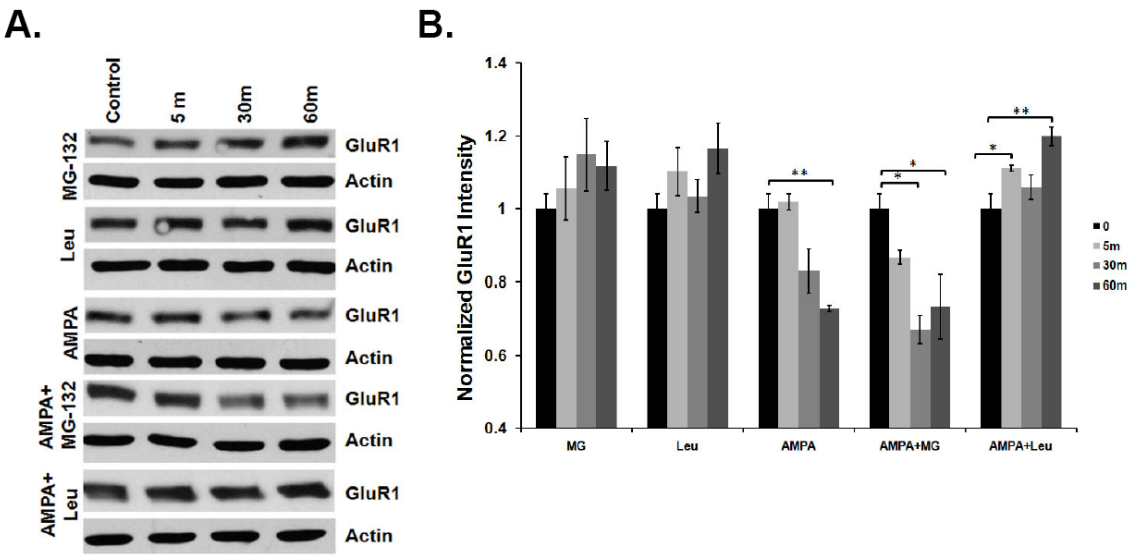

Figure 2-3 The stability of AMPARs after short-term application of AMPA is dependent on lysosomal activity

(A) Dissociated hippocampal cultures were treated with the proteasome inhibitor MG-132 (25 µM) or the lysosome inhibitor leupeptin (200 µg/ml) in combination with AMPA (100 µM) for increasing time points as indicated. Representative Western blot from 3 experiments where cells were lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-GluR1 or anti-actin antibodies. (B) Quantification of (A) over 3 experiments where total GluR1 band intensity from treated lysates was measured and normalized to control lysates. *$p<0.05$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m.

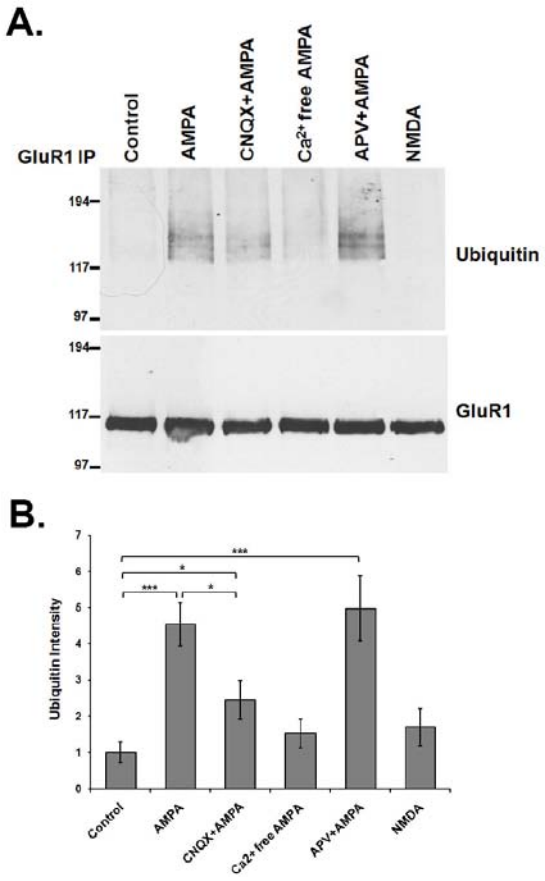

Figure 2-4 Ubiquitination of GluR1-containing AMPARs is dependent on AMPAR activation and calcium

(A) Dissociated neuronal cultures were treated with AMPA (100µM), CNQX (40µM)+AMPA, Ca^{2+}free media+AMPA, APV (50µM)+AMPA, NMDA (25µM), or left untreated prior to IP with anti-GluR1 antibodies. IPs were resolved by Western blot and probed with anti-ubiquitin and anti-GluR1 antibodies. (B) Quantification of normalized mean ubiquitin. n=4-8 IPs for each condition. *$p<0.05$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m.

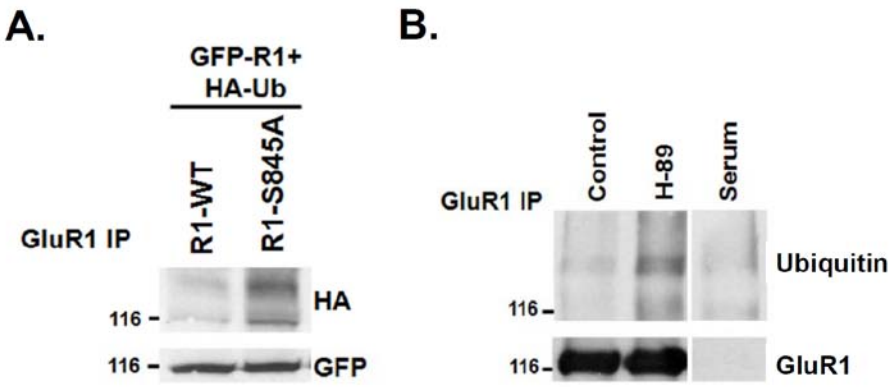

Figure 2-5 GluR1 ubiquitination increases if phosphorylation at serine 845 is inhibited

(A) HEK293T cells were transfected with GFP-tagged GluR1-WT or GluR1-S845A and HA-tagged ubiquitin for 24 hours before performing IPs with anti-GFP antibodies and resolving IPs by Western blot. This Western blot is representative of 3 independent experiments that provided similar results. (B) Mature dissociated neurons were treated with H-89 (2µM, 4hr) or left untreated prior to lysis and IP with anti-GluR1 antibodies. IPs were resolved by Western blot and probed with anti-ubiquitin and anti-GluR1 antibodies. This Western blot is representative of 3 independent experiments.

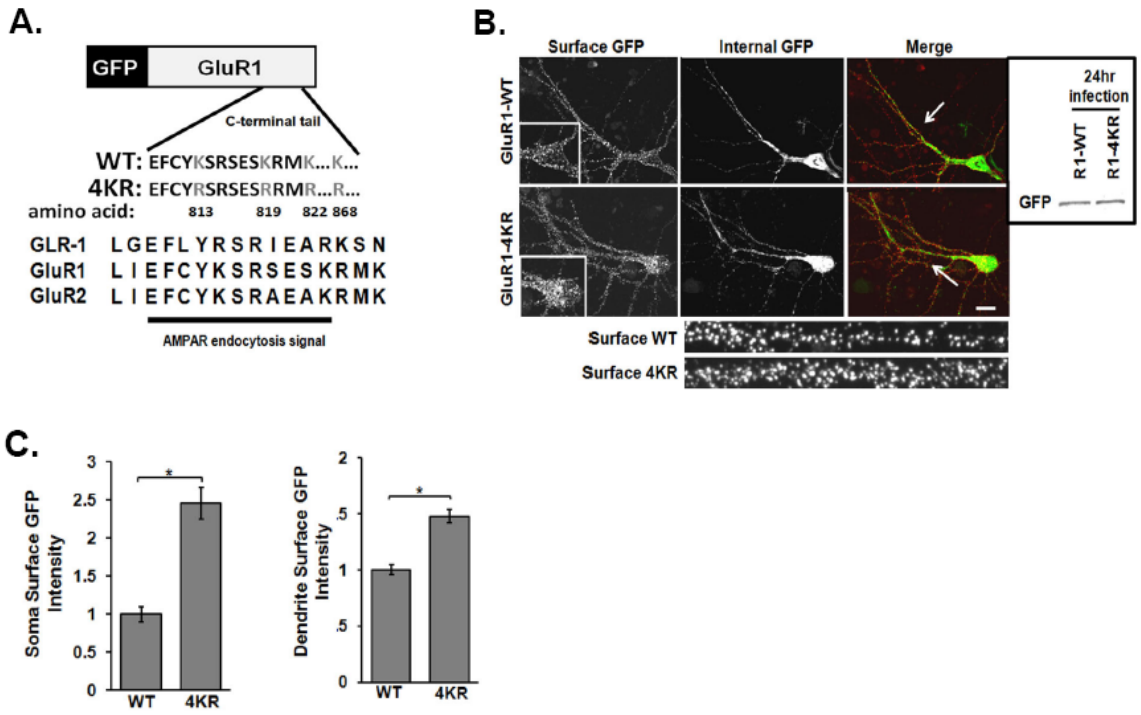

Figure 2-6 Loss of C-terminal ubiquitination sites causes an accumulation of surface GluR1 in neurons

(A) (top panel) A schematic of GFP-tagged GluR1 C-terminal highlights lysine residues that are potential sites of ubiquitination. GFP-GluR1-4KR has all four C-terminal lysines mutated to arginines. (bottom panel) An amino acid sequence in the C-terminal region of GluR1 is similar to *C. elegans* GLR-1, which undergoes ubiquitination, and a region of mammalian GluA2 that contains an endocytosis signal. (B) Representative images of dissociated hippocampal neurons infected with GluR1-WT or GluR1-4KR virus. Surface (red) and internal (green) GFP-GluR1 populations were discretely labeled with anti-GFP antibodies. n=28 cells for WT and n=30 cells for 4KR over 4 experiments. (C) Quantification of (B) over 5 experiments. n=40-50 cells per condition. *$p<0.001$, unpaired Student's t-test.

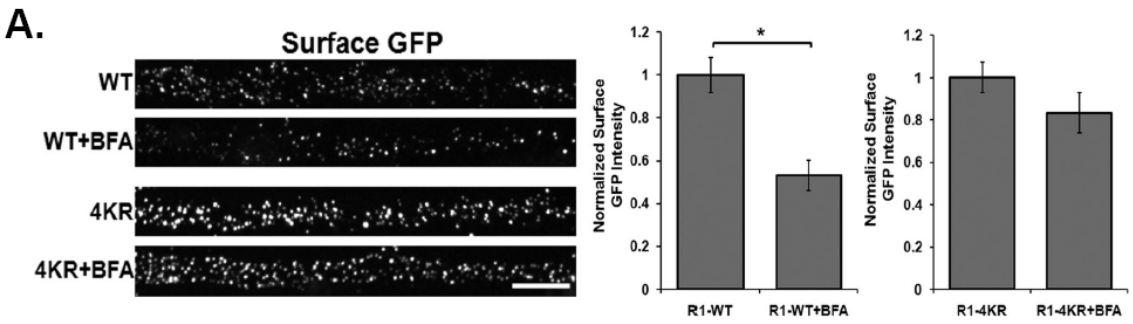

Figure 2-7 Loss of GluR1 C-terminal lysines alters GluR1 surface stability

(A) Representative images of straightened dendrites from hippocampal neurons expressing GFP-GluR1-WT or GFP-GluR1-4KR. Neurons were treated for 45 min. with BFA (5µg/ml) before surface labeling with anti-GFP antibodies. Quantification of (A) over 3 experiments. n=40-50 cells per condition. *$p<0.001$, unpaired Student's t-test. Error bars=s.e.m. Scale bar=10µm.

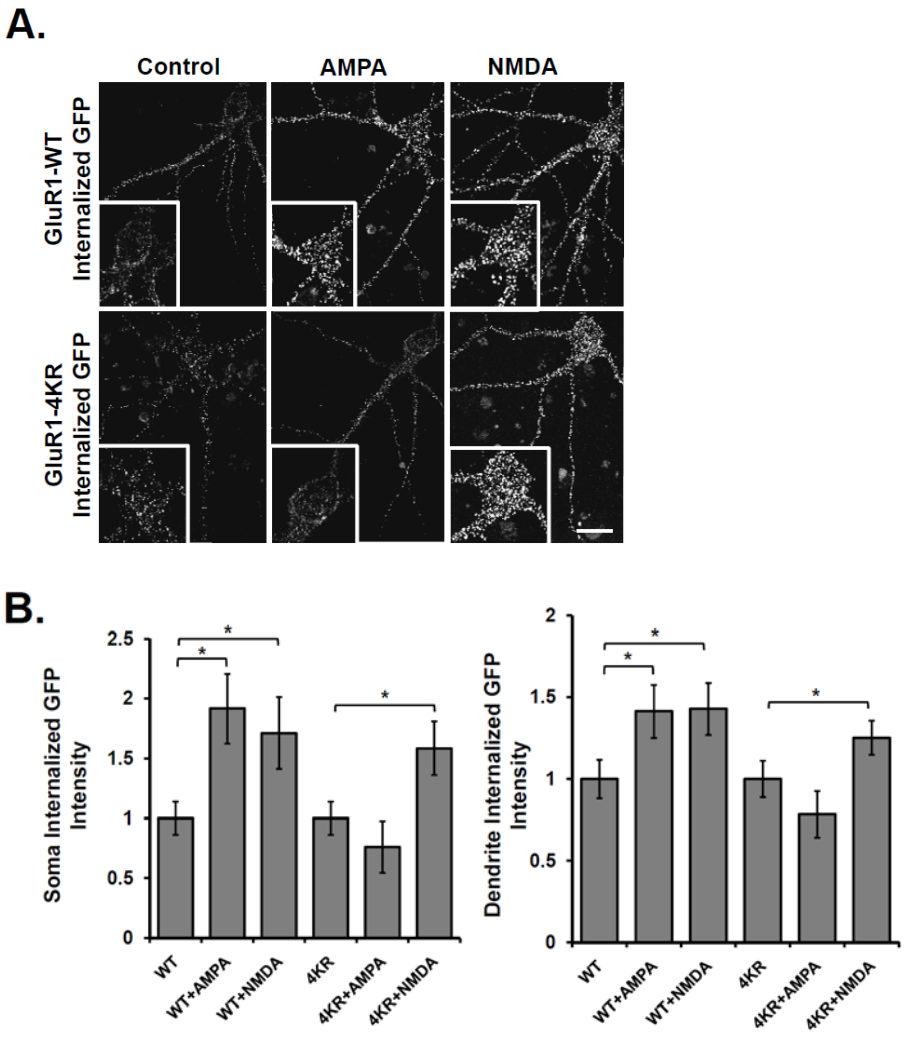

Figure 2-8 Ubiquitination at C-terminal sites is necessary for AMPA-mediated but not NMDA-mediated GluR1 endocytosis

(A) Representative images of internalized GluR1-WT or GluR1-4KR in neurons treated with AMPA (100µM) or NMDA (25µM) for 5-10min. (B) Quantification of internalized GluR1-WT or GluR1-4KR intensity in soma or dendrites of infected neurons. n=30-45 cells per treatment over 4 experiments. *$p<0.05$, ANOVA with Fisher's LSD *post hoc* test. Error bars=s.e.m. Scale bar=10µm.

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III. The E3 Ligase Nedd4-1 Mediates Activity-Dependent Ubiquitination, Endocytosis, and Lysosomal Trafficking of GluR1

Introduction

The process of protein ubiquitination is highly regulated in eukaryotic cells in large part due to the specificity E3 ligases have for their target proteins. There are hundreds of E3 ligases in eukaryotic cells, originating mainly from two families of ligases: RING (really interesting novel gene) ligases and HECT (homology to E6-AP carboxy terminus) ligases [1, 2]. These families of ligases differ in how they attach ubiquitin to target proteins. HECT ligases contain a catalytic cysteine residue that accepts ubiquitin from the E2 and then transfers it to specific lysines on target proteins. Alternatively, RING ligases facilitate an interaction between the E2 and the target protein, but do not directly attach ubiquitin to the target protein themselves.

Characterization of Nedd4 Ligases

The neural-precursor cell-expressed developmentally down-regulated gene 4 (Nedd4) family of ubiquitin ligases are HECT E3 ligases originally identified by their high expression in the embryonic mouse central nervous system [3, 4]. The Nedd4 ligases are present in all eukaryotes from yeast to mammals, and share a common structure: an N-terminal calcium dependent phospholipid binding C2 domain, multiple WW domains that bind to proline-rich regions of proteins, and the HECT domain (Figure 3-1).

C2 Domains

Approximately one hundred proteins have been found to contain C2 domains, with most of these proteins functioning in cellular signal transduction or membrane trafficking pathways [5]. The C2 domain is approximately 130 amino acids and serves as a Ca^{2+}-binding motif that mediates interactions between a variety of ligands and substrates, such as Ca^{2+}, phospholipids, and intracellular proteins. C2 domains were first identified as a conserved domain in several isoforms of the mammalian Ca^{2+}–dependent protein kinase C (PKC) which was not present in Ca^{2+}-independent isoforms [6-9]. C2 domains have also been shown to be important for the function of the neuronal protein Synaptotagmin I. Synaptotagmin I associates with synaptic vesicles in neurons, and contains two C2 domains in its cytoplasmic domain. It is thought that these domains regulate Synaptotagmin's association with the phospholipid membrane of vesicles in the presence of Ca^{2+} to assist in vesicle exocytosis from the pre-synaptic terminal of neurons [10]. The C2 domain of Nedd4 has also been found to bind phospholipids in a Ca^{2+}–dependent manner. In this case, the C2 domain of Nedd4-1 was found to regulate a re-distribution of the protein from the cytosol to plasma membrane of cells in the presence of ionomycin and Ca^{2+} [11]. Also, in yeast the Nedd4 homolog Rsp5 was unable to ubiquitinate or sort target proteins destined for the multivesicular endosome and vacuole if its C2 domain was removed [12]. Though the mechanisms of C2-mediated Nedd4 activation and trafficking have yet to be well studied, a recent paper hypothesized that binding of Ca^{2+} to the C2 domain actually disrupts an interaction between the C2 and HECT domain of Nedd4 ligases, releasing an auto-inhibition of the protein and allowing it to ubiquitinate target proteins [13].

Role of Nedd4-1 in Protein Trafficking

With the discovery of many Nedd4 family ligases and their target substrates, as well as research focusing on the role of Nedd4-mediated ubiquitination on target protein function, trafficking, and stability, it has become clear that the Nedd4 family members are mainly involved in ubiquitination processes unrelated to proteasomal degradation. Rather, they seem to play similar roles across many eukaryotic cell types, mediating ubiquitin-mediated endocytosis, endocytic sorting, and regulation of protein trafficking in the trans-Golgi network of cells.

The yeast homolog of Nedd4-1, Rsp5p, is thought to be the primary E3 ligase in yeast responsible for mediating the endocytosis and endocytic sorting of many plasma membrane proteins [14]. Its presence in yeast is critical, as loss of the rsp5 gene is lethal [15]. One major protein that Rsp5p ubiquitinates, Ste2p, is a G-protein coupled receptor. Interestingly, the region of Ste2p targeted by Rsp5p for ubiquitination shares sequence similarity to the C-terminal tail of mammalian AMPARs [16]. *C. elegans* and *D. melanogaster* each have three Nedd4 family members, which target several proteins to mediate a variety of cellular functions, such as embryo development, axon guidance, and Notch signaling [17-19].

In mammalian cells, the number of Nedd4 family members increases, as does the diversity of their target proteins and their roles in cellular function. Interestingly though, many of these Nedd4 family members, as well as their substrates, have been found to play an important role in the nervous system. The founding member of the Nedd4 family, Nedd4-1, was originally discovered due to its high expression in the mouse

embryonic central nervous system [20]. Since then, it has been shown that Nedd4 family members target voltage-gated Na^+ channels, Trk neurotrophin receptors, dopamine transporters, voltage-gated K^+ channels, and glutamate transporters [21-25].

Since Nedd4 ligases, particularly Nedd4-1, have an established role in ubiquitin-mediated endocytosis in eukaryotic cells and have also been found to perform this function in neurons, we were interested in exploring a potential relationship between Nedd4-1 and AMPAR ubiquitination in hippocampal neurons.

Results

Nedd4-1 is Present in Mature Hippocampal Synaptic Fractions

While Nedd4-1 was first discovered due to its high expression during neural development, its expression profile has not been well characterized in the mature hippocampus. To do this, we resolved hippocampal culture lysates ranging in age from 3 to 23 days in vitro (DIV) by SDS-PAGE and probed the Western blot with anti-Nedd4-1 or anti-GluR1 antibodies (Figure 3-2 A). We found Nedd4-1 to be heavily expressed in immature cultures, and its expression persisted at a moderate level throughout development. As expected, we observed that GluR1 was present in the cultures after DIV7, and increased in expression through DIV23.

We also performed a biochemical fractionation of mature rat brain tissue to further investigate the neuronal location of Nedd4-1 (Figure 3-2 B). Nedd4-1 was present in post-synaptic densities, suggesting that it may reside at or near synapses in the

developed brain. Importantly, this data suggests that Nedd4-1 may be located in post-synaptic compartments where AMPARs reside.

Nedd4-1 Ubiquitinates GluR1 and Decreases Surface GluR1 Levels When Co-Expressed in HEK293T Cells

Since Nedd4 family members have previously been shown to be involved in ubiquitin-mediated endocytosis in many eukaryotic cell types, including neurons, we were interested to establish if they played a role in AMPAR ubiquitination. We first tested whether Nedd4-1 was involved in regulating the trafficking of GluR1. GFP-tagged GluR1-WT and HA-tagged Nedd4-1 were co-transfected in HEK293T cells. Cells were live-labeled with an anti-GFP antibody, permeabilized, labeled with an anti-HA antibody, and imaged by confocal microscopy. Co-expression of Nedd4-1 dramatically decreased surface GluR1 levels (1±0.08 for GluR1-WT to 0.3±0.03 for GluR1-WT+Nedd4-1)(Figure 3-3 A,B). Co-expression of GluR1 with other HECT domain ligases (Nedd4-2 and E6AP) as well as a RING finger ligase (Cbl) did not decrease surface GluR1 levels (Figure 3-3 C). The ability of Nedd4-1 to reduce surface GluR1 levels required its ligase activity, since co-expression of a catalytically-inactive version of Nedd4-1 (Nedd4-1 CS) did not decrease surface GluR1 levels (Figure 3-3 A,B). Importantly, we found that Nedd4-1 had no effect on surface GluR1 populations when co-transfected with GluR1-4KR (Figure 3-3 A,B). It also did not affect surface GluR2 levels when co-expressed (Figure 3-3 C). Ubiquitination of surface GluR1-WT by Nedd4-1 was also confirmed biochemically. HEK293T cells were co-transfected with GFP-tagged GluR1-WT or GluR1-4KR, HA-tagged ubiquitin, and Nedd4-1. After 24 hours, these cells were surface

labeled with anti-GFP antibody, lysed, precipitated to isolate the antibody-labeled surface GluR1, and resolved by SDS-PAGE. HA-ubiquitin immunoreactivity occurred most abundantly when GluR1-WT was co-expressed with Nedd4-1, while GluR1-4KR showed minimal ubiquitination, even in the presence of Nedd4-1 (Figure 3-4 A). GluR1 ubiquitination by Nedd4-1 was further confirmed by co-expressing these proteins in HEK293T cells, isolating GluR1 by immunoprecipitation, and visualizing ubiquitin reactivity of the receptor by Western blot. Using the anti-ubiquitin antibody P4D1, we detected increased ubiquitination of GluR1 when co-expressed with Nedd4-1, but a loss of GluR1 ubiquitination when co-expressed with Nedd4-1 CS (Figure 3-5 A, B).

GluR1 and Nedd4-1 Directly Interact in HEK293T Cells and Hippocampal Tissue

We also observed a specific interaction between GluR1 and Nedd4-1 when co-expressed in HEK293T cells, while no interaction between Nedd4-1 and GluR2 or NR1 was observed (Figure 3-6 A, B, D). Furthermore, GluR1 did not interact with a highly similar Nedd4 ligase family member, Nedd4-2 (Figure 3-6 C). These observations suggest that an interaction between Nedd4-1 and GluR1 is specific and likely direct, as HECT ligases have been shown to interact directly with their targeted substrates. Additional evidence that endogenous GluR1 and Nedd4-1 interact in mature hippocampal neurons came from immunoprecipitation of Nedd4-1 from lysates of mature hippocampal tissue (Figure 3-7). When we resolved these IPs on Western blots and probed them with anti-GluR1 antibodies, we found GluR1 to be present in the Nedd4-1 precipitates. Similarly, when we immunoprecipitated GluR1 from mature hippocampal lysates, we found Nedd4-1 to be present in the GluR1 precipitates (Figure 3-7). Together, these

results indicate that Nedd4-1 associates with GluR1, providing an opportunity for it to ubiquitinate and regulate the trafficking of GluR1-containing AMPARs in hippocampal neurons.

Over-expression of Nedd4-1 Leads to Loss of Surface and Synaptic GluR1-containing AMPARs

Since over-expression of Nedd4-1 led to a significant decrease in surface GluR1 in HEK293T cells (Figure 3-3) and Nedd4-1 associates with AMPARs in hippocampal neurons (Figure 3-7), we reasoned that over-expression of Nedd4-1 in hippocampal neurons would diminish surface AMPAR populations in those neurons. Neurons, DIV 15-18, were infected with Sindbis virus expressing either GFP alone (control) or co-expressing GFP with HA-tagged Nedd4-1. Infection time was limited to 18-22 hours to limit cell toxicity. Cells were then labeled with antibodies directed against surface GluR1, permeabilized, and labeled with anti-HA antibodies to detect Nedd4-1 positive cells. Compared to uninfected neurons or GFP-control cells, neurons expressing HA-Nedd4-1 showed a significant loss in surface GluR1 immunofluorescence from the dendritic plasma membrane (1±0.03 for GFP, 0.7±0.03 for Nedd4-1, *$p<0.001$,unpaired Student's t-test) indicating that increased expression of Nedd4-1 decreased surface populations of GluR1-containing AMPARs (Figure 3-8 A). We demonstrated this result was not caused by increased insertion of newly synthesized receptors by treating infected hippocampal neurons with BFA (5µg/ml, 45min) (Figure 3-9 A). After BFA treatment, there was a significant loss in surface GluR1 immunofluorescence in control neurons (1±0.05 for GFP to 0.7±0.04 for GFP+BFA). Untreated Nedd4-1-infected neurons had

less surface GluR1 than control neurons, as expected (1±0.05 for GFP to 0.7±0.04 for Nedd4-1). After BFA treatment, however, there was an even larger decrease in surface GluR1 immunofluorescence (0.7±0.04 for Nedd4-1 to 0.5±0.05 for Nedd4-1+BFA). This suggests that over-expression of Nedd4-1 leads to increased endocytosis or decreased recycling of GluR1-containing AMPARs. This loss was specific to Nedd4-1 because over-expression of another HECT ligase, E6-AP, did not change surface GluR1 levels (Figure 3-10 A).

To determine if the Nedd4-1-induced decrease in surface AMPARs affected synaptic AMPARs, we recorded spontaneous miniature excitatory postsynaptic currents (mEPSCs) from GFP (control) or Nedd4-1 infected neurons. We observed a significant decrease in mEPSC amplitude in neurons expressing Nedd4-1 compared to control neurons (23.4±1.4 pA for GFP, 17.6±1.3 pA for Nedd4-1)(Figure 3-11 A-D) while the mEPSC frequency was not significantly different between conditions (0.69±0.24 s inter-event interval (IEI) for GFP, 0.59±0.15 s for Nedd4-1)(Figure 3-11 E). Also, mEPSC amplitude recordings from neurons infected with catalytically inactive Nedd4-1 (Nedd4-1 CS) were unchanged from control neurons (Figure 3-10 C). We confirmed similar expression of HA-Nedd4-1 WT and Nedd4-1 CS in infected neurons by immunostaining infected neurons with anti-HA antibodies (Figure 3-10 D). This suggests that over-expression of Nedd4-1 in hippocampal neurons causes a decrease in surface AMPARs populations that includes synaptic AMPARs.

Because our data suggest GluR1 ubiquitination mediates AMPAR trafficking to the lysosome, we hypothesized that over-expression of Nedd4-1 may cause increased

trafficking of surface AMPARs to the lysosome. To test this, we briefly surface labeled hippocampal neurons with anti-GluR1 antibodies prior to the addition of GFP or Nedd4-1-expressing Sindbis virus. To one set of infected neurons, we also applied leupeptin (200µg/ml) to block lysosomal degradation. After 18-22 hours, we fixed the neurons, blocked any remaining surface GluR1 antibody with unconjugated secondary antibodies, permeabilized the cells, and labeled the population of internalized GluR1. GFP-expressing neurons, with or without leupeptin, showed minimal levels of internalized GluR1 immunofluorescence (Figure 3-12 A, B). However, in neurons expressing Nedd4-1 where lysosomal degradation was inhibited by leupeptin, there was a dramatic accumulation of internalized GluR1 in both the soma and dendrites. This accumulation did not occur in Nedd4-1-infected neurons if leupeptin was not added (1.8±0.18 for N4-1+leu to 1±0.13 for control, 1±0.07 for control+leu, and 0.9±0.10 for N4-1). A significant portion of internalized GluR1 puncta co-localized with late-endosomal/lysosomal compartments, visualized with anti-Lamp1 antibodies, in Nedd4-1 expressing neurons when compared to control neurons (2.8±0.45 for N4-1+leu to 1±0.22 for control+leu and 0.9±0.12 for N4-1+leu with random Lamp1 signal)(Figure 3-12 C). These results indicate that expression of Nedd4-1 in hippocampal neurons targets endogenous AMPARs to late endosomal/lysosomal compartments.

Loss of Nedd4-1 Inhibits AMPA- but not NMDA-Mediated Endocytosis of GluR1-Containing AMPARs

We additionally explored the function of Nedd4-1 in regulating GluR1 endocytosis and endocytic sorting by examining the effects of RNAi-mediated

knockdown of Nedd4-1 in mature hippocampal neurons. We designed a small hairpin RNA (shRNA) directed against Nedd4-1 that sufficiently knocked down expression of Nedd4-1 protein compared to scramble or empty vector controls (Figure 3-13). We also generated a version of Nedd4-1, called Nedd4-1resist., which was resistant to RNAi-mediated knockdown. Nedd4-1resist. also contained a T7 tag which was used to confirm its expression in cells. Neurons were transfected at DIV10 with a control vector (pSuper-GFP or pSuper-Scramble) or Nedd4-1 shRNA (pSuper-RNAi). After 4-5 days of expression, neurons were labeled with antibodies specific to surface GluR1, imaged by confocal microscopy, and analyzed for any changes in surface GluR1 populations. We observed no change in surface GluR1 immunofluorescence or synaptic GluR1 levels detected by mEPSC recordings (data not shown). Our previous findings suggested, however, that ubiquitination of GluR1 occurs under conditions when GluR1-containing AMPARs are directly activated and endocytosis is induced. Therefore, we hypothesized that loss of Nedd4-1 may prevent the internalization of AMPARs in response to AMPA. To test this, we examined AMPA-induced internalization of AMPARs in control and Nedd4-1 shRNA-expressing hippocampal neurons. As expected, AMPA produced robust internalization of GluR1 in control neurons (1±0.10 for control to 1.6±0.19 for control+AMPA)(Figure 3-13 B, C). In contrast, Nedd4-1 shRNA-expressing neurons showed a significant inhibition of AMPA-induced AMPAR internalization (0.7±0.12 for RNAi and 0.5±0.07 for RNAi+AMPA). Strikingly, RNAi-expressing neurons were still able to internalize GluR1 in response to NMDA at levels similar to control neurons exposed to NMDA (1±0.10 for RNAi to 1.3±0.10 for RNAi+NMDA and 1.4±0.10 for control+NMDA)(Figure 3-13 B, D). Importantly, co-expression of pSuper-RNAi with

Nedd4-1resist. rescued the neurons' ability to endocytose GluR1-containing AMPARs in response to AMPA (1±0.12 for Nedd4-1resist. to 1.4±0.16 for Nedd4-1resist.+AMPA,*$p<0.05$, unpaired Student's t-test)(Figure 3-13 B, E). This indicates that Nedd4-1 mediates AMPAR internalization following direct AMPAR activation, but is not required for NMDA-dependent endocytosis, and suggests that Nedd4-1 is crucial for mediating AMPAR internalization and trafficking to the lysosome.

AMPA-Mediated GluR1 Ubiquitination is Dependent on Nedd4-1 and Increases as Neurons Mature

To further confirm a direct role for Nedd4-1 in AMPA-mediated GluR1 ubiquitination, we created a lentivirus expressing Nedd4-1 RNAi, which allowed us to knockdown endogenous Nedd4-1 expression in large populations of dissociated neuron cultures. The virus also expressed GFP to allow for identification of infected neurons. We infected neurons at DIV9 with Nedd4-1 RNAi lentivirus or lentivirus expressing GFP alone as a control. At DIV14, neurons were treated with AMPA (100µM, 10min.), lysed, and GluR1 was isolated by immunoprecipitation (Figure 3-14). While AMPA induced significant ubiquitination of GluR1 in DIV14 control neurons as previously observed (Figure 2-1), ubiquitination of GluR1 was completely abolished in cultures lacking Nedd4-1 (3.0±0.4 for DIV14 AMPA to 1±0.2 for DIV14 control and 1.3±0.4 for DIV14 RNAi+AMPA). While performing these experiments, we observed that in older neurons (DIV35), GluR1 was significantly ubiquitinated under control conditions, at levels similar to ubiquitination of GluR1 induced by application of AMPA in younger neurons (3.4±1 for DIV35 control and 2.8±0.7 for DIV35 AMPA to 3.0±0.4 for DIV14 AMPA).

Furthermore, this increase in GluR1 ubiquitination in older neurons was still dependent on the presence of Nedd4-1, as loss of Nedd4-1 due to expression of Nedd4-1 RNAi lentivirus for five days resulted in significantly decreased GluR1 ubiquitination levels similar to that of younger untreated neurons (1.±0.3 for DIV35 RNAi+AMPA to 1±0.2 for DIV14 control). These results suggest that factors, such as increased AMPAR activation or Nedd4-1 ligase activity, may change in neurons as they age, resulting in increased Nedd4-1 mediated AMPAR ubiquitination.

Materials and Methods

Antibodies and Reagents: Antibodies were obtained as follows: pAb GluR1, pAb GluR2, pAb Nedd4-1 (Millipore), pAb surface (N-terminal) GluR1 (Calbiochem), mAb ubiquitin (P4D1) and pAb GFP (Santa Cruz), anti-GFP secondary antibody (Invitrogen), pAb Actin (Cytoskeleton Inc.), AMPA and NMDA (Tocris), BFA (Invitrogen), N-ethylmaleimide (NEM) and leupeptin (Sigma), MG-132 (Biomol).

Brain Fractionation: Mature rat brain was homogenized in HEPES-buffered sucrose (.32 M sucrose, 4mM HEPES, pH 7.4) and homogenized. The homogenate (Homog.) was centrifuged at 1000 X g at 4°C to remove the nuclear fraction (P1). The supernatant was centrifuged at 10,000 X g for 15 min to isolate the crude synaptosomal pellet (P2). The P2 pellet was lysed in cold H_2O plus protease inhibitors and homogenized, followed by resuspension to 4mM HEPES. Lysates were centrifuged at 25,000 X g for 20 min to produce a supernatant (S3) and pellet (P3). The P3 was resuspended in HEPES-buffered sucrose, layered over a sucrose gradient, and centrifuged at 150,000 X g for 2 hr. The synaptic plasma membrane was recovered, resuspended in 50mM HEPES, 2mM EDTA,

.5% TX-100, plus protease inhibitors, and centrifuged at 32,000 X g for 20 min to obtain the PSD-1T. A fraction of the PSD-1T pellet was resuspended and centrifuged as described above to obtain the PSD-2T pellet.

Hippocampal Cultures: Rat dissociated hippocampal or hippocampal and cortical cultures from postnatal day 1 were plated onto poly-D-lysine coated coverslips, 35 mm dishes (Mattek), or 6 well plastic dishes and maintained in B27 supplemented Neurobasal media (Invitrogen) until 14-38 days in vitro.

Immunoprecipitations: Rat hippocampal tissue or hippocampal cultures were homogenized in precipitation buffer (PB) (in mM: 150 NaCl, 10 Na_2HPO_4, 2 EDTA) with 1% TX-100 and 0.1-0.2% SDS, 25 µM MG-132, 25mM NEM, and protease inhibitors. HEK293T cells were lysed in RIPA Buffer plus 1% BSA with 25 µM MG-132, 25mM NEM, and protease inhibitors. Homogenates were cleared by centrifugation at 14,000 rpm at 4°C. For immunoprecipitations, cleared lysates were incubated with primary antibodies at 4°C O/N, after which protein A or protein A/G sepharose beads were added for an additional 2 hours (Pierce). Immunoprecipitations from neurons were repeated 3-8 times. Immunoprecipitations from HEK293T cells were repeated 2-4 times. For all immunoprecipitations from transfected cells, equal protein expression was verified in cell lysates by Western blot. For quantification of Western blots, protein band mean intensities were calculated using ImageJ. For Western blots measuring protein ubiquitination, ubiquitin band mean intensities were divided by the mean intensities of the corresponding immunoprecipitated receptor. Ubiquitination values from treated IPs were normalized to values from control IPs. For Western blots measuring GluR1 levels,

the GluR1 band mean intensity in each condition was normalized to the actin band mean intensity from the same sample.

Transfections and Infections: HEK293T cells, maintained in DMEM + 10% serum and Pen/Strep, were transfected with Lipofectamine 2000 (Invitrogen) or PEI (Polysciences) using recommended protocols. Hippocampal cultures were infected with Sindbis virion at DIV15-19 and allowed to express for 18-22 hours. For RNAi experiments, hippocampal cultures were transfected with Lipofectamine 2000 at DIV10 and expressed the RNAi constructs for 4-5 days, or infected with lentivirus expressing the RNAi constructs for 5 days. Viral titer and transduction efficiency were monitored for all viruses made to ensure equal expression of constructs.

DNA Constructs: GFP-GluR1 obtained from R. Malinow (UCSD) was mobilized in pcDNA3.1(-) vector. GluR1-4KR, Nedd4-1 CS, and Nedd4-1resist. were created using PCR site-directed mutagenesis. All point mutations were verified by sequencing. For Sindbis viral expression, genes were cloned into the Sindbis virus vector SinRep5. For lentiviral expression, an H1 promoter and RNAi sequence were cloned into the FG-12 vector expressing GFP. HA-Nedd4-1 and HA-Cbl plasmids were purchased from Addgene DNA Depository. HA-E6-AP was obtained from P. Howley (Harvard), T7-Nedd4-1 was obtained from D. Rotin (Sick Kids), myc-NR1-1a and GFP-GluR2 was obtained from A. Ghosh (UCSD), and YFP-Nedd4-2 was obtained from S. Polo (FIRC Institute).

Surface Live-labeling and Endocytosis: Dissociated hippocampal neurons (DIV15-20) or HEK293T cells were live-labeled with anti-GFP secondary or anti-GluR1 antibodies for

15 min at 37°C, and then washed with PBS-MC (1X PBS, 1mM $MgCl_2$, 0.1mM $CaCl_2$). Cells were then fixed for 5-10 min. with 4% paraformaldehyde/4% sucrose at room temperature. Surface GluR1 receptors were then labeled with secondary Alexa antibodies (Molecular Probes) in PBS-MC containing 2% BSA. For endocytosis experiments, cells were pre-treated with TTX (2 µM) for 1 hour before surface labeling. After surface labeling, cells were washed with PBS and replaced with conditioned media containing either vehicle or AMPA (100 µM) and APV (25µM), or NMDA (25µM) and glycine (20µM) for 10-15 min. at 37°C. Cells were then washed with PBS-MC and fixed. Surface receptors were labeled with untagged secondary antibodies in PBS-MC before the cells were permeabilized with PBS-MC, 2% BSA, and 0.2% Triton X-100 for 20 min. The internalized receptors were labeled with fluorophore-conjugated secondary antibodies. For endocytosis experiments involving co-staining with Lamp1 antibody, cells were permeabilized in PBS containing 2% NGS, 1% BSA, and 0.1% saponin for 1 hour at RT. For endocytosis experiments, the fluorescence from the GluR1-N terminal GFP molecule was negligible post fixation as its fluorescence was determined to be detectable only at exposure times ten times greater than those used to detect antibody-labeled GFP.

Colocalization: To quantify the amount of internalized GluR1 immunofluorescence co-localized with Lamp1 immunostaining, images of Lamp1-labeled dendrites were converted to thresholded masks using ImageJ. Thresholded images of internalized GluR1-labeled dendrites were pasted over these masks and the remaining intensity of internalized GluR1 signal was quantified. To verify that colocalization was specific,

colocalization between internalized GluR1 and Lamp1 in Nedd4-1+leu cells was calculated as described above where Lamp1 staining was first randomized by rotating the image 180° before creating a mask.

RNAi: To knockdown expression of Nedd4-1 in hippocampal neurons, the oligo GCCACAAATCAAGAGTTAA was synthesized and inserted into the pSuper-eGFP vector or the FG-12 vector. A scramble oligo (GCAGACAAACCTATGAATA) was also created. Dissociated neuronal cultures were transfected with pSuper-Nedd4-1 or infected with FG-12-Nedd4-1 RNAi at DIV10 and experiments were conducted 4-5 days later. To create Nedd4-1resist., six silent point mutations were introduced into T7-Nedd4-1 using PCR mutagenesis into the region targeted by RNAi.

Electrophysiology of dissociated hippocampal neurons: For recording of miniature excitatory postsynaptic currents (mEPSCs) from GFP, GFP + Nedd4-1 WT, GFP + Nedd4-1 CS, and shRNA-expressing hippocampal neurons, cells were perfused at room temperature in a bicarbonate buffered recording solution ((in mM) 124 NaCl, 5 KCl, 26 NaHCO3, NaH2PO4, 2 MgCl2, 3 CaCl2, 0.5 TTX, 20 picrotoxin, 10 glucose) bubbled constantly with 95% O2/5% CO2. The intracellular solution contained (in mM): 10 CsCl, 105 CsMeSO3, 0.5 ATP, 0.3 GTP, 10 HEPES, 5 glucose, 2 MgCl2, 1 EGTA, 0.2 QX-314, pH 7.3. Electrode resistance ranged from 4 to 7 MΩ, access resistances ranged from 10 to 25 MΩ and were monitored for consistency throughout the recordings. Cells with a leak current >100 pA were excluded from analysis. Signals were amplified, filtered to 2 or 5 kHz and digitized at 10 kHz sampling frequency. All recordings were taken from time points after mEPSC frequency and amplitude had reached steady state, greater than

10 min following perfusion of TTX and picrotoxin. mEPSCs were analyzed using custom software in Igor Pro or MiniAnalysis.

Confocal Microscopy and Image Analysis: All images were taken with a Leica DMI6000 inverted microscope outfitted with a Yokogawa Nipkon spinning disk confocal head, an Orca ER high resolution B&W cooled CCD camera (6.45 μm/pixel at 1X), Plan Apochromat 40X/1.25na and 63X/1.4na objective, and an argon/krypton 100mW air-cooled laser for 488/568/647 nm excitations. All images were acquired in the dynamic range of 8 bit or 12 bit acquisition. Maximum projected confocal Z-stacks were analyzed with NIH ImageJ. For experiments analyzing surface or internalized GFP or GluR1 immunofluorescence, images were background subtracted and thresholded equally, and the integrated density of each puncta was measured using a modified ImageJ particle analysis macro. The average particle integrated density for each cell was normalized to cell size. This raw data value for each cell was divided by the average raw data value of untreated control cells to obtain a normalized value. Values from the same treatments over multiple experiments were then combined and averaged.

Statistical Analysis: Two-tailed unpaired Student's *t* test with an alpha=.05 or ANOVA with Tukey's or Fisher LSD *post-hoc* analysis was used for determining statistical significance. Results >0.05 were not considered significant.

Acknowledgements: The electrophysiological experiments described in Figure 3-11 were done in collaboration with Dr. Benjamin Hall while he was a post-doctoral fellow in the lab of Dr. Anirvan Ghosh.

A.

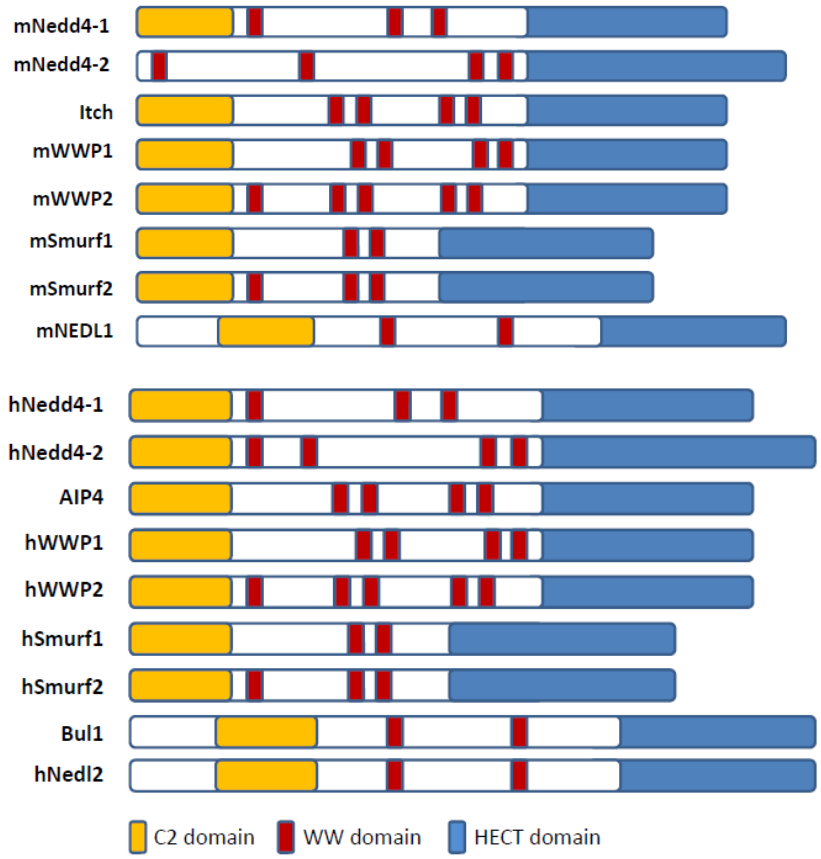

Figure 3-1 Comparison of mouse and human Nedd4 ligase family members

(A) Nedd4 ligases are members of the HECT (homology) family of E3 ligases. All Nedd4 family members contain multiple WW domains, which assist in protein-protein interactions through binding of proline-rich motifs. All Nedd4 family members, except for mNedd4-2, also have a C2 domain, which interacts with Ca^{2+} and phospholipids in a Ca^{2+}-dependent manner. Adapted from: Ingham et al., *The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture.* Oncogene, 2004. **23**(11):1972-84.

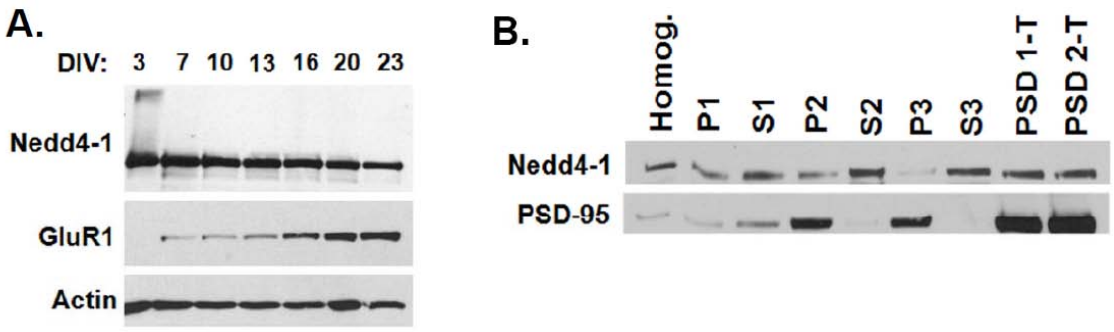

Figure 3-2 Nedd4-1 is present in mature hippocampal neurons at the post-synaptic density

(A) Dissociated neuronal cultures of increasing age were lysed and resolved by Western blot before probing with anti-Nedd4-1, anti-GluR1, or anti-actin antibodies. (B) Dissociated neuronal cultures were fractionated via centrifugation to purify post-synaptic density fractions before resolving samples on a Western blot and probing with anti-Nedd4-1 or anti-PSD-95 antibodies.

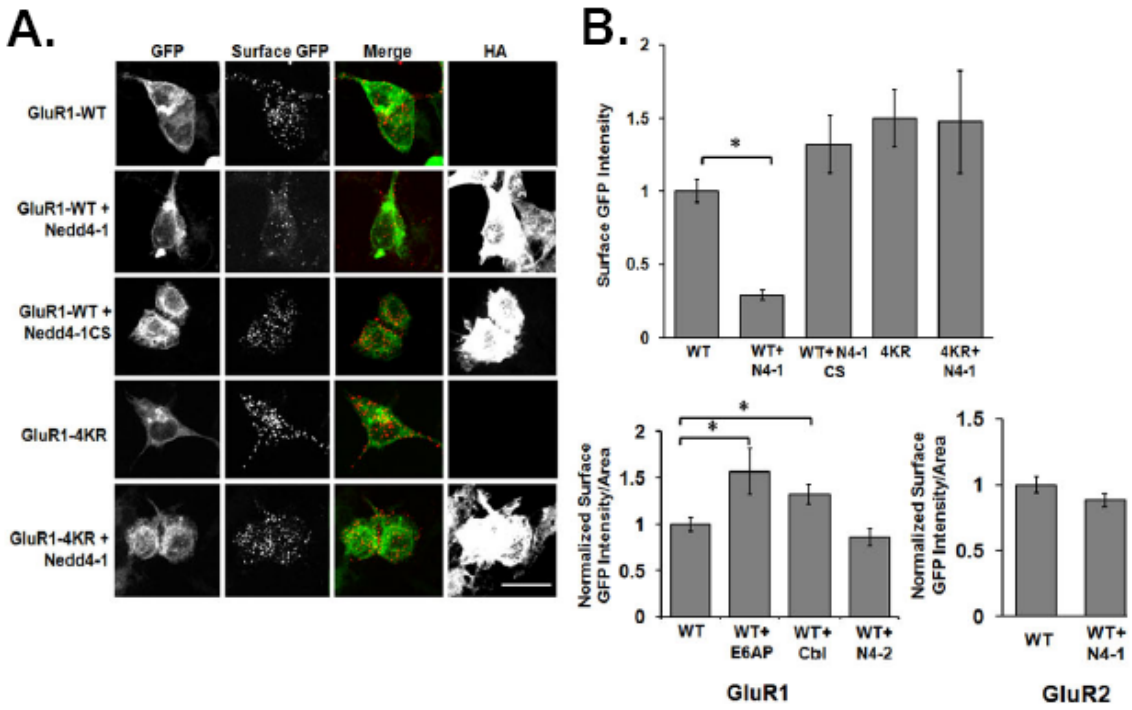

Figure 3-3 Nedd4-1 reduces surface AMPAR levels when co-expressed in HEK293T cells

A) Representative images of HEK293T cells transfected with GFP-GluR1-WT or 4KR alone, or with HA-tagged Nedd4-1 or catalytically-inactive Nedd4-1 (Nedd4-1 CS), and surface labeled with anti-GFP antibodies. Scale bar=10µm. (B) Quantification of surface GFP intensity over 3 experiments where HEK293T cells were transfected with GFP-tagged GluR1-WT, GluR1-4KR, or GluR2 and HA-tagged E3 ligases. n=30-40 cells per condition. *p<0.01, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m.

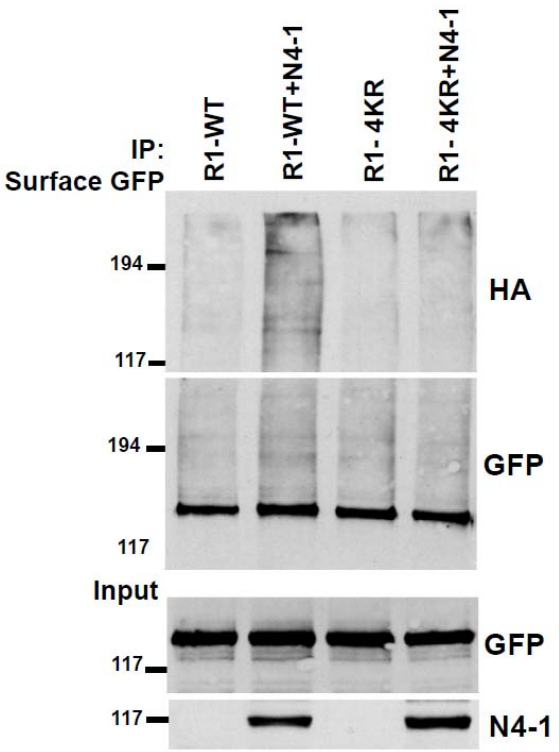

Figure 3-4 Nedd4-1 ubiquitinates GluR1 at C-terminal lysines when co-expressed in HEK293T cells

(A) Representative Western blot where HEK293T cells co-transfected with HA-ubiquitin and GluR1-WT or GluR1-4KR alone or with Nedd4-1. They were then surface labeled with anti-GFP antibodies prior to lysis of cells and IP to isolate surface labeled receptors. IPs were resolved by Western blot and probed with anti-HA antibodies to visualize ubiquitination. n=2 IPs per condition.

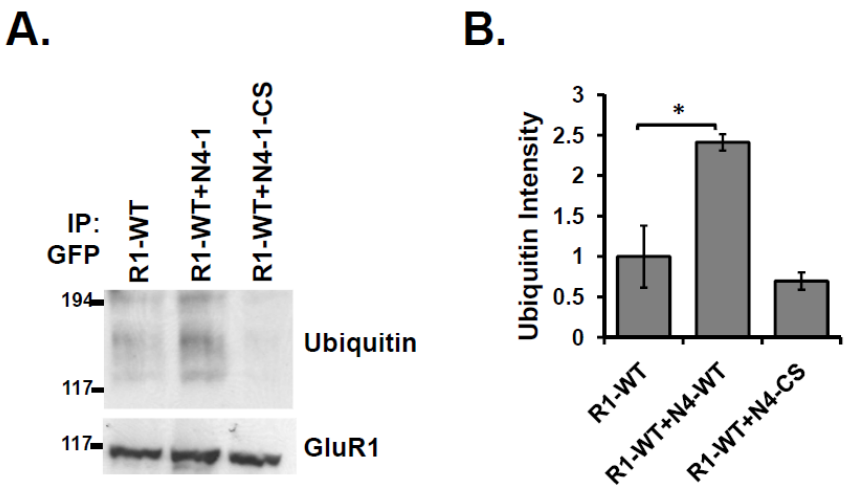

Figure 3-5 Co-expression with Nedd4-1 causes an increase in GluR1 ubiquitination in HEK293T cells

(A) Representative Western blot where HEK293T cells co-transfected GluR1-WT and Nedd4-1 or a version of Nedd4-1 that is catalytically inactive (Nedd4-1-CS). GluR1 was isolated by IP, resolved by Western blot, and probed with anti-ubiquitin antibodies to visualize ubiquitination. n=3 IPs per condition. (B) Quantification of GluR1 ubiquitination over 3 experiments. *$p<0.05$, unpaired Student's t-test. Error bars=s.e.m.

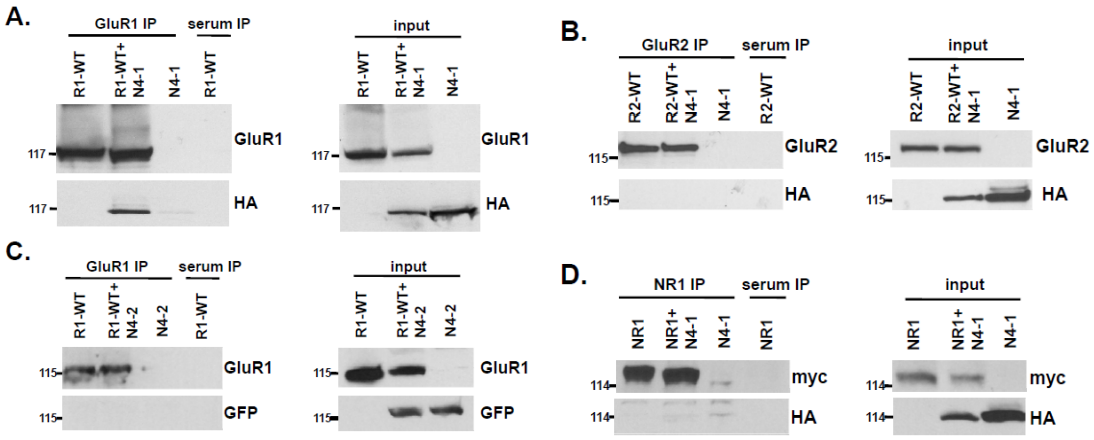

Figure 3-6 The interaction between GluR1 and Nedd4-1 is specific when co-expressed in HEK293T cells

(A) Representative Western blot from 3 experiments of GluR1 IPs from HEK293T cells co-expressing GFP tagged GluR1 and HA-tagged Nedd4-1. (B) Representative Western blot from 2 experiments of GluR2 IPs from HEK293T cells co-expressing GFP tagged GluR2 and HA-tagged Nedd4-1. (C) Representative Western blot from 3 experiments of GluR1 IPs from HEK293T cells transfected with untagged GluR1-WT alone or GluR1-WT and YFP-Nedd4-2. (D) Representative Western blot from 2 experiments of NR1 IPs from HEK293T cells co-expressing myc-tagged NR1 and HA-tagged Nedd4-1.

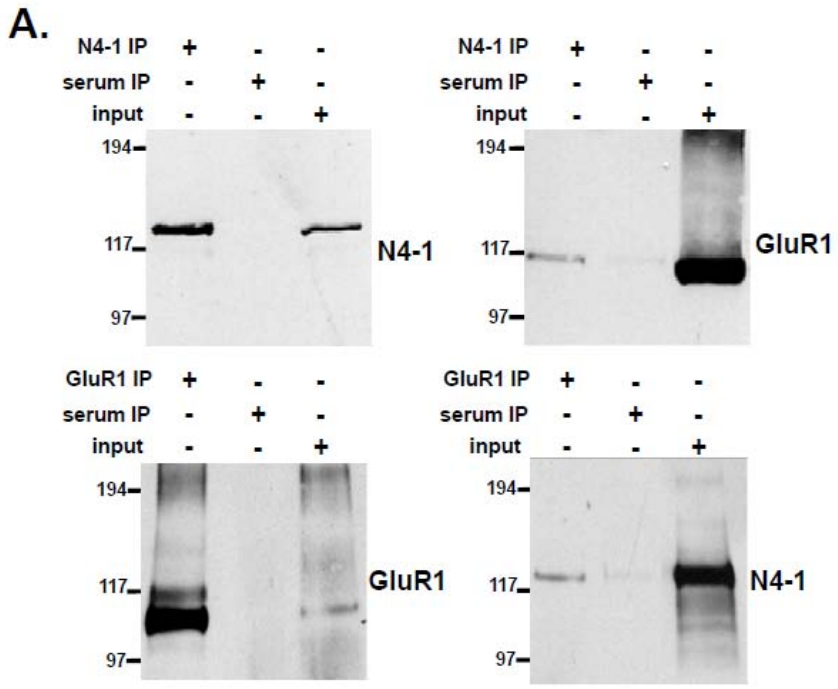

Figure 3-7 Nedd4-1 interacts with GluR1 in hippocampal tissue

(A) Nedd4-1 or GluR1 was isolated via IP with anti-Nedd4-1 or anti-GluR1 antibodies from mature hippocampal tissue. IPs were resolved by Western blot and probed with anti-GluR1 or anti-Nedd4-1 antibodies. IPs were repeated 3 times.

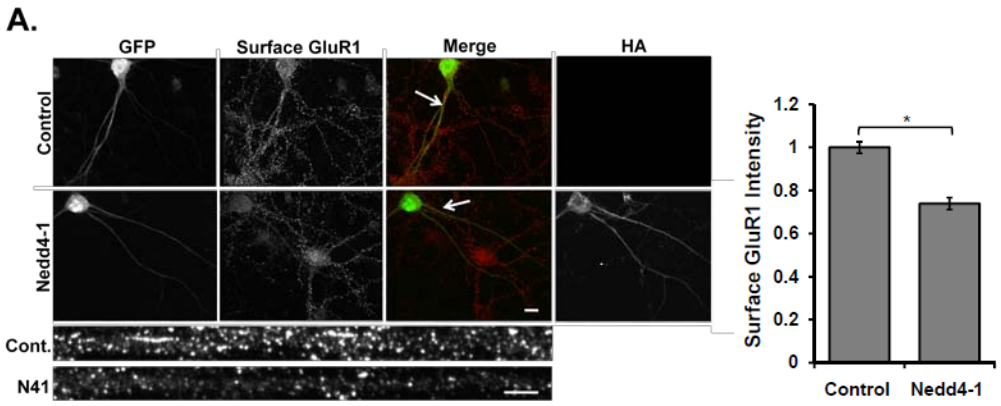

Figure 3-8 Nedd4-1 regulates surface expression of GluR1

(A) Representative images of neuronal cultures infected with GFP virus or GFP and HA-Nedd4-1 virus and surface-labeled with anti-GluR1 antibodies. Quantification of surface GluR1 intensity in dendrites of infected neurons was performed. n=50 cells for control, n=65 cells for Nedd4-1, over 6 experiments. Scale bar=10μm for whole cell images, 5μm for straightened dendrites. *$p<0.05$, unpaired Student's t-test.

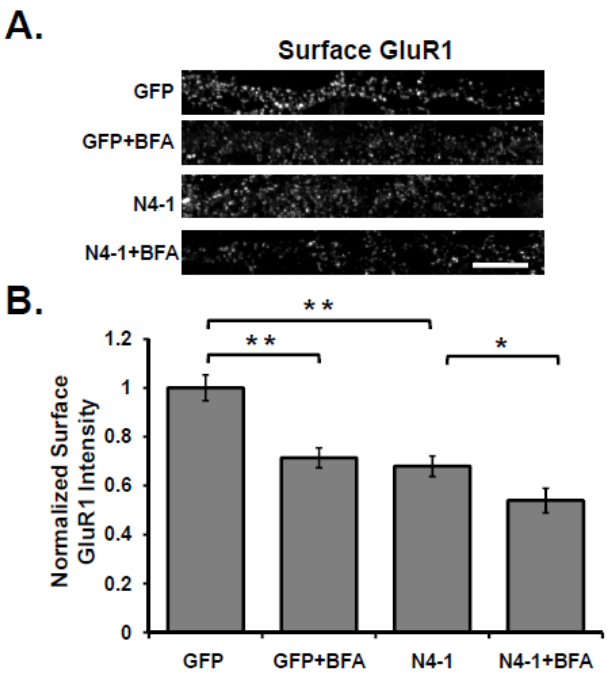

Figure 3-9 Over-expression of Nedd4-1 alters GluR1 surface stability

(A) Representative images of straightened dendrites from hippocampal neurons expressing GFP or GFP and HA-Nedd4-1. Neurons were treated for 45 min. with BFA (5µg/ml) before surface labeling with anti-GluR1 antibodies. (B) Quantification of (A) over 2 experiments. n=25-35 cells per condition. *p<0.05, **p<0.01, ANOVA with Tukey's *post hoc* test. Error bars s.e.m. Scale bar=5 µm for straightened dendrites.

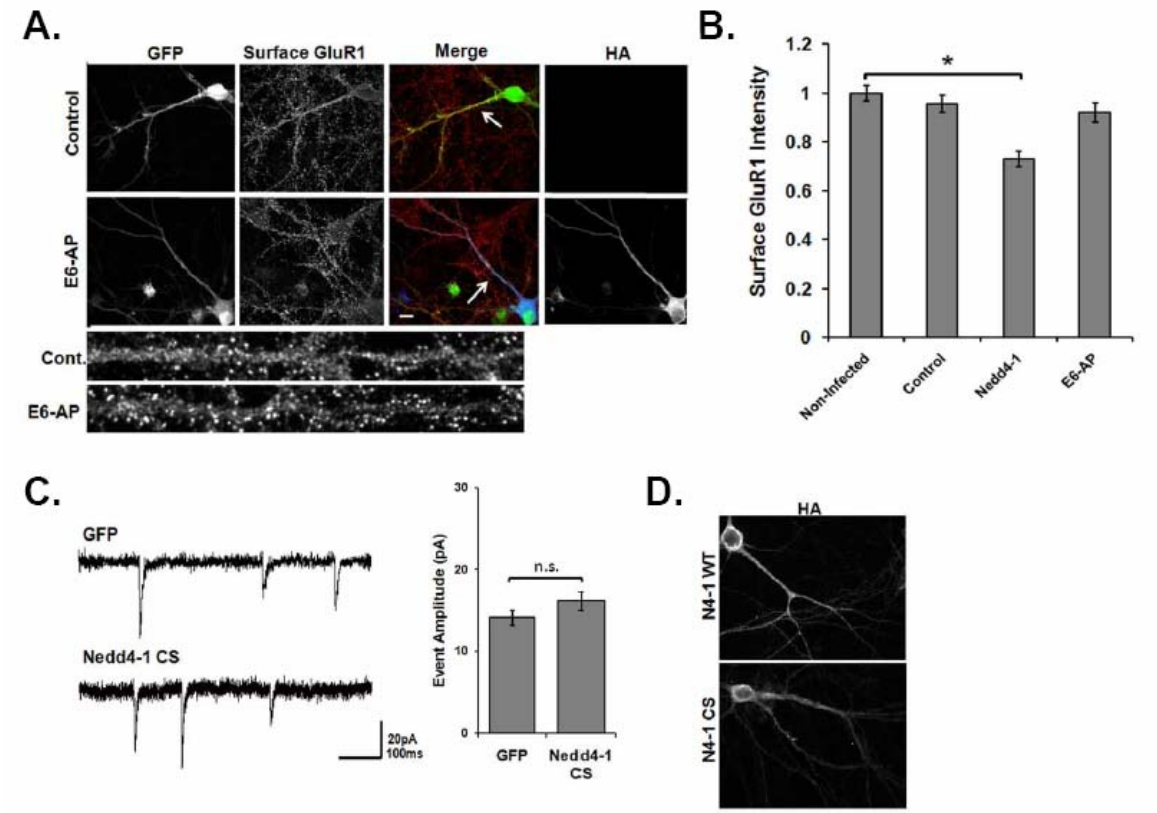

Figure 3-10 Over-expression of the E3 ligase E6-AP or Nedd4-1 CS in hippocampal neurons has no effect on surface GluR1

(A) Representative images of hippocampal neurons infected with Sindbis virion expressing GFP or GFP and HA-tagged E6-AP, and surface labeled with anti-GluR1 antibodies. Scale bar= 10 µm for whole cell images, 5 µm for straightened dendrites. (B) Quantification of surface GluR1 immunofluorescence of non-infected neurons from coverslips where neighboring neurons were infected with GFP, Nedd4-1, or E6-AP Sindbis virus. n=35-45 cells per condition over 3 experiments. *$p<0.05$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m. (C) Example mEPSC traces recorded from GFP or Nedd4-1 CS infected neurons. Quantification of event amplitudes averaged over all cells expressing either GFP (control) or Nedd4-1 CS shows that Nedd4-1 has no effect on mEPSC amplitude. n=16 cells for control, n=18 cells for Nedd4-1 CS over 7 experiments. Error bars=s.e.m. (D) Representative image of neurons infected with HA-tagged Nedd4-1 WT or Nedd4-1 CS and immunostained with anti-HA antibodies. Both viruses expressed similar levels of HA-Nedd4-1 protein in neurons.

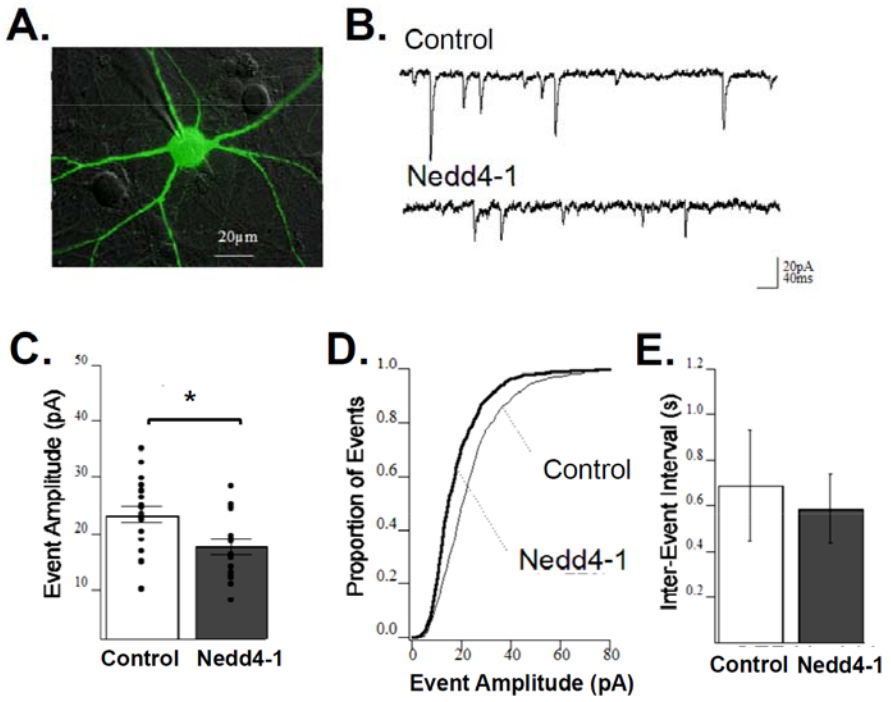

Figure 3-11 Nedd4-1 regulates synaptic GluR1 populations

(A) Representative image of a neuron expressing GFP and Nedd4-1 during mEPSC recording. (B) Example mEPSC traces recorded from GFP (control) or Nedd4-1 infected neurons. (C) Quantification of event amplitudes averaged over all neurons expressing either GFP (control) or Nedd4-1. *$p<0.005$, unpaired Student's t-test. (D) Cumulative histogram of event amplitudes for GFP (control) or Nedd4-1 neurons. (E) Quantification of event frequencies averaged over all neurons expressing GFP (control) or Nedd4-1. n=21 cells for control, n=19 cells for Nedd4-1 over 6 experiments. Error bars=s.e.m.

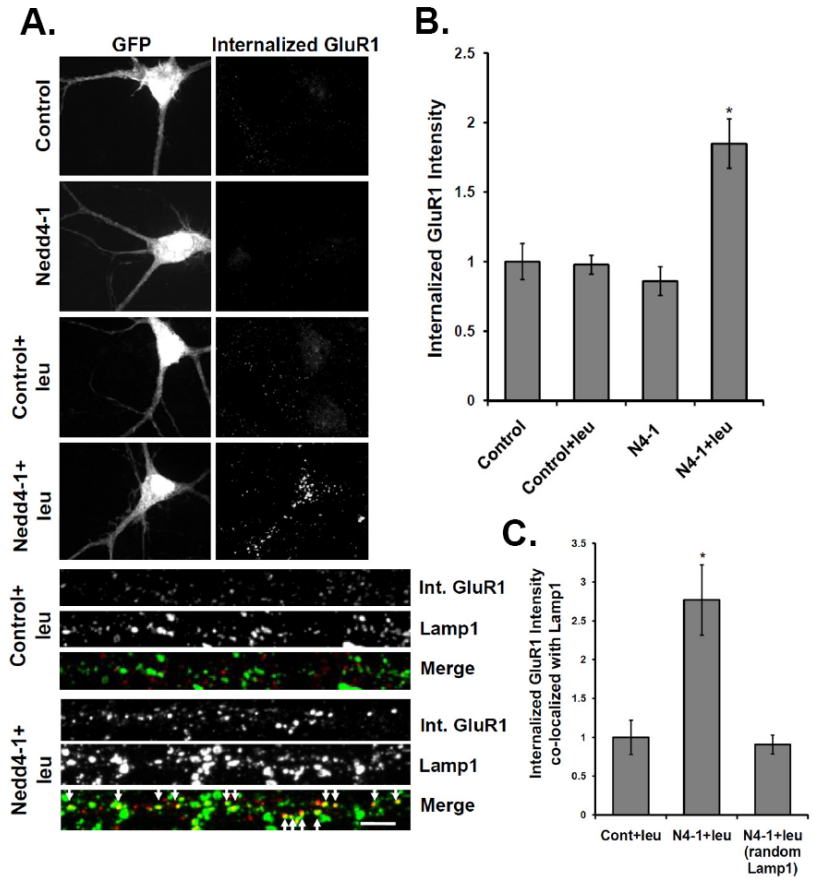

Figure 3-12 Over-expression of Nedd4-1 causes increased trafficking of surface GluR1-containing AMPARs to the lysosome

(A) Neuronal cultures were surface-labeled with anti-GluR1 antibodies prior to infection with GFP (control) or Nedd4-1 virus, in the absence or presence of leupeptin. Representative images of internalized GluR1 (red) in infected neurons 18-22 hours post-infection and straightened dendrites co-labeled with the late endosome/lysosome antibody Lamp1 (green). Scale bar=10μm for whole cell images, 5μm for straightened dendrites. (B) Quantification of internalized GluR1 intensity in dendrites of infected neurons. (C) Quantification of internalized GluR1 intensity co-localized with Lamp1 staining in dendrites of infected neurons. n=30-45 cells per condition over 3 experiments. *$p<0.05$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m.

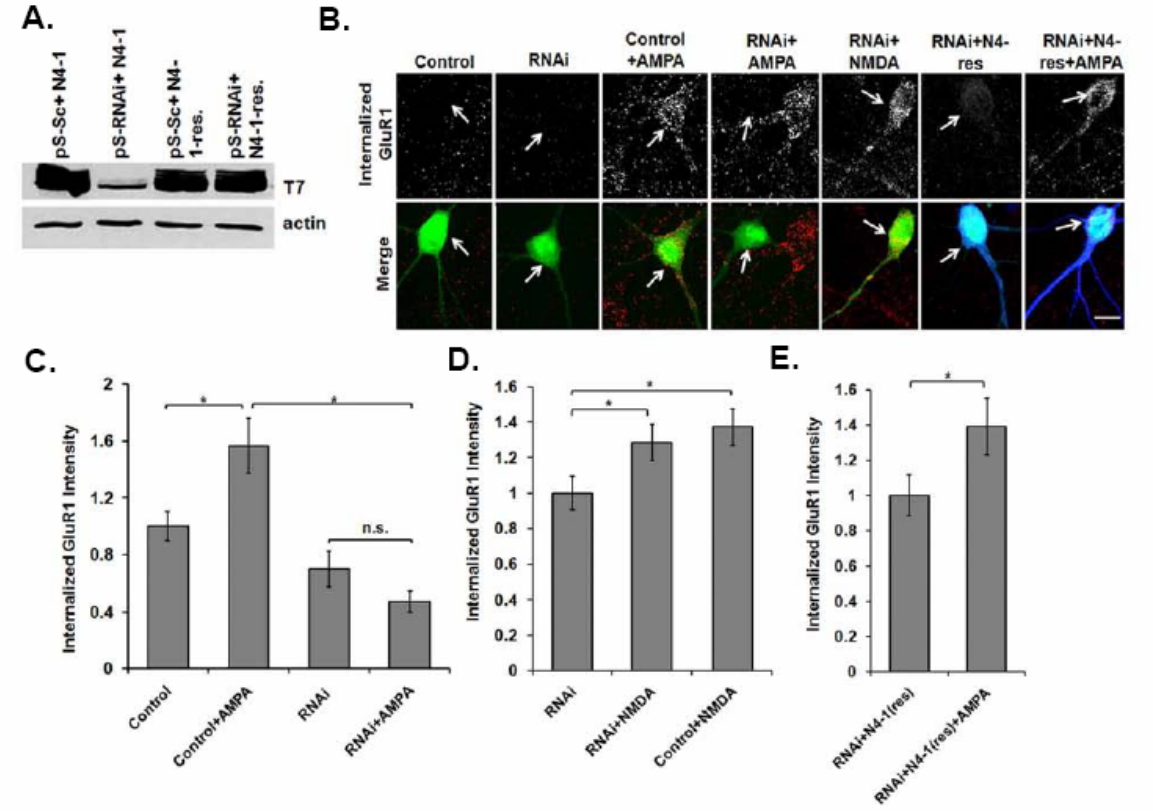

Figure 3-13 Loss of Nedd4-1 inhibits AMPA-mediated but not NMDA-mediated endocytosis of GluR1-containing AMPARs

(A) Representative Western blot of lysates from HEK293T cells co-transfected with T7-Nedd4-1 or Nedd4-1resist. and pSuper-scramble or pSuper-Nedd4-1-RNAi. (B) Transfected neurons were surface-labeled prior to a 10 min. application of AMPA (100µM) or NMDA (25µM). Representative images are of internalized GluR1 (red) after each treatment in neurons expressing control or Nedd4-1 RNAi vectors (green) alone, or with Nedd4-1resist. (blue). Scale bar=10µm. (C-D) Quantification of internalized GluR1 intensity in control or RNAi-transfected neurons treated with AMPA (C) or NMDA (D). (E) Quantification of internalized GluR1 intensity in neurons transfected with Nedd4-1 RNAi and Nedd4-1resist. and treated with AMPA (100µM) for 10 min. n=30-40 cells per condition over 3-4 experiments. *p<0.05. Error bars=s.e.m. *p<0.05, ANOVA with Tukey's *post hoc* test (C,D) or unpaired Student's t-test (E). Error bars=s.e.m.

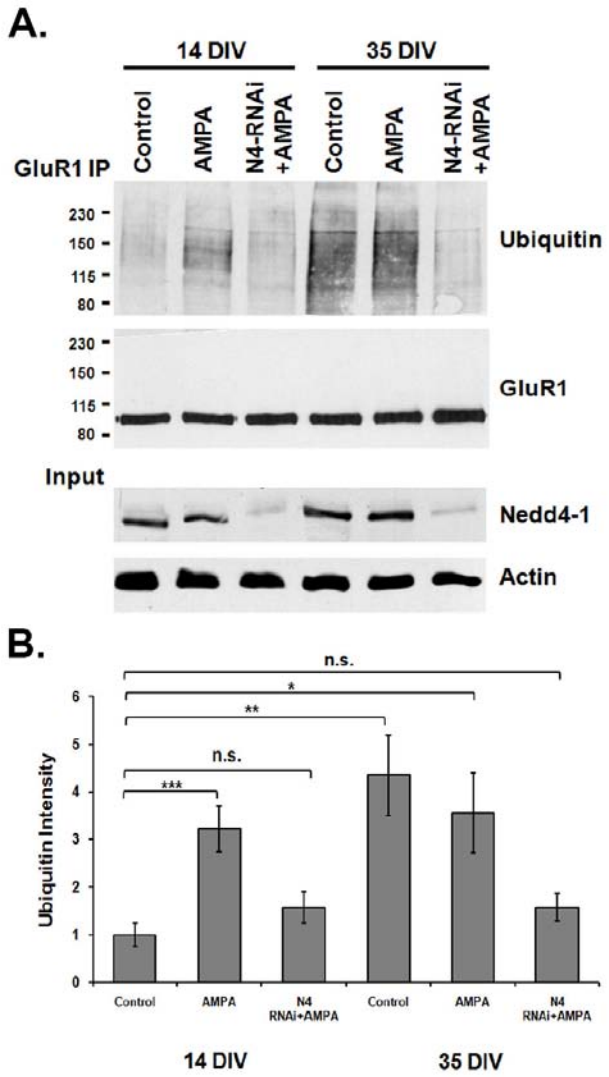

Figure 3-14 Ubiquitination of GluR1-containing AMPARs is up-regulated in aged neurons but blocked by loss of Nedd4-1

(A) Dissociated neuronal cultures (DIV14 or DIV35) infected with lentivirus expressing GFP or GFP and Nedd4-1 RNAi for five days were treated with AMPA (100µM, 10min.) or left untreated prior to immunoprecipitation of resulting lysates with anti-GluR1 antibodies. IPs were resolved by Western blot and probed with anti-ubiquitin antibodies and antibodies against GluR1 to confirm equal levels of protein in each IP. Lysates were also resolved and probed with anti-Nedd4-1 and anti-actin antibodies to confirm Nedd4-1-specific knockdown. (B) Quantification of mean ubiquitin intensity for each IP. n=4-6 IPs for each condition. *$p<0.05$, **$p<0.01$, ANOVA with Tukey's *post hoc* test. Error bars=s.e.m.

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IV. Conclusion

In this thesis, I have reported that direct activation of GluR1-containing AMPARs induces their ubiquitination. This process is not dependent on NMDAR activation, but does require calcium. Ubiquitination occurs on C-terminal lysines of GluR1 and is mediated by the E3 ligase Nedd4-1. Ubiquitination at these sites mediates the endocytosis and lysosomal trafficking of GluR1-containing AMPARs, but may not be required for their endocytosis and recycling back to the plasma membrane (Figure 4-1).

While our data shows that ubiquitination of GluR1 by Nedd4-1 is required for its endocytosis under specific conditions, it is likely that other proteins assist in this process. Indeed, in many eukaryotic cell types, the interaction between ubiquitination and clathrin machinery is a well studied and common occurrence in the regulation of endocytosis and sorting of proteins [1]. Ubiquitinated receptors can be recruited to clathrin-coated pits via interactions with the adaptor proteins epsin and eps15 [2]. Also, ubiquitination of receptors may aid their interaction with the adaptor protein AP2 [3]. Both epsin and AP2 have characterized roles in neurons for regulating protein endocytosis [4-7]. Furthermore, GLR-1, the *C. elegans* homolog of GluR1, has been shown to undergo ubiquitination and removal from the synapse in manner dependent on the clathrin adaptor protein AP180 [8]. However, it's also intriguing to speculate that distinct machinery could regulate ubiquitin-dependent and independent AMPAR internalization. Indeed, this phenomenon has been observed for the EGF receptor (EGFR). Depending on the level of ligand exposure and receptor activation, EGFRs can undergo varying amounts of ubiquitination which differentially regulate their entrance into clathrin-dependent or

independent endocytosis pathways [1]. It is interesting to hypothesize that the relative activation of AMPARs and NMDARs could differentially recruit clathrin and ubiquitination machinery to regulate the specificity of endocytosis and endocytic sorting of AMPARs.

Our studies are the first to show that GluR1-containing AMPARs undergo ubiquitination in mature hippocampal neurons, and through multiple experiments, we have identified Nedd4-1 as the E3 ligase responsible for mediating GluR1 endocytosis and sorting to the lysosome. Interestingly, two other studies have recently shown that Nedd4-1 is crucial for neuronal development [2, 3]. Specifically, Kawabe et al. created both a traditional and conditional Nedd4-1 knockout mouse to identify that Nedd4-1, the serine/threonine kinase TNIK, and Rap2A form a complex to mediate Rap2A ubiquitination and ultimately dendrite formation. Several key differences in the methodologies of Kawabe et al. and our experiments strongly suggest that we have uncovered a distinct and novel role for Nedd4-1 in mature hippocampal neurons that may be distinct from its role in neuronal development. A significant difference is that Kawabe et al. focused on the role of Nedd4-1 very early in neuronal development, while we examined Nedd4-1's role in mature neurons. Indeed, Nedd4-1 expression is highly upregulated during nervous system development before stabilizing to moderate levels in mature neurons, suggesting that its role in neurons may change over time [4, 5]. In addition, several neuronal proteins have already been identified as Nedd4-1 targets, again indicating that Nedd4-1 most likely has several distinct roles in neurons [6, 7]. In fact, Kawabe et al. show that Nedd4-1 associates with TNIK and Rap2A in the perinuclear region of neurons, and that these three proteins co-segregate with the Golgi and ER.

However, our studies identify a role for Nedd4-1 in mediating AMPAR endocytosis from the plasma membrane of the soma and dendrites of neurons, in support of our findings that Nedd4-1 is important for balancing surface and synaptic AMPARs populations in mature neurons. These differences point out the interesting possibility that Nedd4-1 has not only temporal and substrate-specificity, but possibly even spatially distinct roles in neurons.

Based on our findings and previous work exploring the activity-dependent sorting of AMPARs, we hypothesize that the relative activation of NMDARs and AMPARs can differentially lead to the recruitment of phosphorylation or ubiquitination machinery which determine the fate of internalized AMPARs [8]. This is an especially appealing hypothesis as Nedd4-1's activity and recruitment to the plasma membrane has been found to be regulated by Ca^{2+} in non-neuronal eukaryotic cells [9, 10]. Therefore, it is plausible that Nedd4-1 may be recruited to ubiquitinate GluR1 during specific Ca^{2+}–dependent signaling events in neurons. If and how Nedd4-1 is activated by Ca^{2+} in neurons is completely unknown, but future experiments exploring this phenomenon would be of great interest.

Our results indicate that AMPA-mediated ubiquitination and lysosomal degradation occur to a population of surface GluR1-containing AMPARs when directly activated by agonist. Meanwhile ubiquitination machinery is not recruited by rapid NMDAR activation, which stimulates a fast, but reversible, removal of synaptic AMPARs. The mechanisms described in this thesis provide new insight into how neurons simultaneously and discretely regulate the endocytosis, recycling, and

degradation of AMPARs. The amount of ubiquitinated AMPARs we were able to detect in our experiments was distinct but small compared to total AMPAR populations in hippocampal neurons. Detection of ubiquitinated proteins is challenging because ubiquitination itself is a rapid and reversible process [11]. Therefore, it is possible that the amount of ubiquitinated GluR1 visible by Western blot in our experiments is an under-representation of ubiquitinated AMPAR populations in neurons. Furthermore, our data suggests that surface AMPARs are targeted for ubiquitination, and it has been reported that only 15-20% of total AMPARs in hippocampal neurons reside at the plasma membrane [12]. In addition, many of these surface AMPARs are recycled back to the plasma membrane in a presumably ubiquitin-independent process, suggesting that AMPAR ubiquitination and lysosomal trafficking may only occur to small populations of AMPARs at any given time [8]. However, we observed that ubiquitination of AMPARs under basal conditions increased as neurons matured. These results suggest that as time passes, factors, such as increased activation of AMPARs or increased Nedd4-1 activity, may change in neurons that cause increased endocytosis and lysosomal degradation of AMPARs.

Our results suggest that AMPARs are ubiquitinated to mediate their removal from the plasma membrane and trafficking to the lysosome for degradation, and that this process may be upregulated as neurons mature. We believe these findings may be of interest in relation to other studies focusing on the molecular mechanisms of age-associated neurodegenerative diseases such as Alzheimer's disease (AD). AD is the most common cause of dementia in elderly individuals, characterized by deficits in learning

and memory due to hippocampal dysfunction with eventual loss of cognitive function in other brain areas [13, 14]. Early studies of AD focused on hallmarks, such as neurofibrillary tangles and beta-amyloid (Aβ) plaques, found in brains of deceased AD patients, with the conclusion that the accumulation of these plaques and tangles are central to the pathogenesis of the disease. However, research in recent years has suggested that learning and memory deficits in AD patients occur before the visible formation of plaques and tangles, suggesting earlier unknown molecular mechanisms may contribute to AD pathogenesis. It now appears that improper processing and accumulation of the peptide Aβ may have several negative effects on synaptic function in early stages of the disease. Aβ is formed when amyloid precursor protein (APP) residing in the plasma membrane of cells undergoes proteolytic processing involving α-, β-, and γ-secretases. One of the products of this cleavage is Aβ, which is secreted from the cell. Aβ is found in the cerebrospinal fluid and plasma of healthy individuals throughout their lives, though its role in regular cellular function is not well understood [15, 16]. However, several papers now suggest that excessive amounts of Aβ protein can have severe effects on synaptic strength in hippocampal neurons. Application of Aβ has been shown to inhibit hippocampal long-term potentiation, as has over-expression of APP in transgenic animals [17-20]. Aβ has also been shown to depress excitatory synaptic activity through mechanisms dependent on NMDAR activity [21]. It is thought that this Aβ-induced synaptic depression is the direct result of a loss of synaptic AMPARs in hippocampal neurons, and suggests that normal AMPAR trafficking may be mutated in these models of AD [22-24]. Separately, it has been shown that neurons expressing APP are impaired in ubiquitin-mediated endosomal/lysosomal trafficking [25]. Specifically,

this paper observed the endosomal trafficking of the epidermal growth factor receptor (EGFR) after application of EGF in neurons also expressing APP. They reported that in APP-expressing neurons, there was increased ubiquitination of EGFRs but impaired degradation. As a result of this finding and others, they concluded that Aβ accumulation impairs both the endocytic and UPS pathway in neurons. Indeed, there are interesting parallels between the research described above and the findings presented in this thesis. Our research has detailed a pathway by which AMPARs, when over-stimulated through application of AMPA or over-expression of the ligase Nedd4-1, are ubiquitinated, resulting in their removal from the synapse and trafficking through the endocytic pathway to the lysosome for degradation. Furthermore, we've presented data suggesting that this pathway becomes more active as neurons mature. Combining these results with the papers cited above, it's intriguing to hypothesize that increased ubiquitination of AMPARs could be linked to some of the deficits observed in AD models. Indeed, further research focusing on the circumstances under which AMPAR ubiquitination occurs will likely provide important insight into our understanding of synaptic function under normal and diseased conditions in neurons.

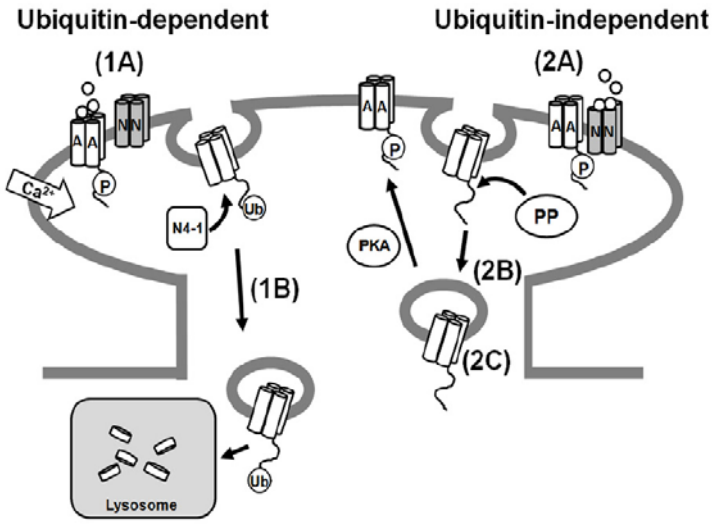

Figure 4-1 Model for ubiquitin-mediated endocytic trafficking of AMPARs

(1A) A distinct endocytosis/lysosomal sorting pathway that is dependent on ubiquitination can be stimulated by direct activation of AMPARs with the agonist AMPA and may be up-regulated during aging. Application of AMPA causes an influx of calcium into hippocampal neurons that may activate the E3 ligase Nedd4-1 to ubiquitinate GluR1-containing AMPARs, most likely in conjunction with phosphatases and other machinery to assist in endocytosis. (1B) Ubiquitination of GluR1-containing AMPARs mediates their trafficking to the lysosome and eventual degradation. (2A) AMPARs can be recruited to a separate endocytosis/recycling pathway upon exposure to NMDA that is not ubiquitin-dependent. (2B) Application of NMDA causes GluR1 to become de-phosphorylated at serine 845 by protein phosphatases (PP) and internalized. (2C) If GluR1 is re-phosphorylated at serine 845 by PKA, it is trafficked to recycling endosomes and returned to the plasma membrane.

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