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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Activity-dependent regulation of ubiquitination and protein degradation controls
excitatory synaptic transmission and plasticity

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Samantha Lynn Scudder

Committee in charge:

Professor Gentry Patrick, Chair
Professor Brenda Bloodgood
Professor Jeffry Isaacson
Professor Yishi Jin
Professor Robert Malinow

2016

The dissertation of Samantha Lynn Scudder is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

I would like to dedicate my dissertation to my parents, Susan Roy and Keith Scudder. Though they've never been able to keep track of what exactly I'm doing, they have provided me with unending support and enthusiasm. They emphasized the importance of a good education and academic excellence early on, and without that I would never be where I am today.

TABLE OF CONTENTS

Signature Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Abbreviations.....	vii
List of Figures.....	ix
Acknowledgments.....	x
Vita.....	xiv
Abstract of the Dissertation.....	xv
Chapter 1: Introduction.....	1
1.1: Types of synaptic plasticity in the central nervous System.....	2
1.2: Posttranslational modifications of glutamate receptors.....	4
1.3: Regulation of AMPAR ubiquitination.....	7
1.4: Function of ubiquitination in AMPAR trafficking.....	9
1.5: AMPAR loss in Alzheimer's disease.....	12
1.6: Role of the ubiquitin-like molecule NEDD8 in the nervous system.....	14
1.7: Fate of ubiquitinated Proteins.....	16
1.8: Proteasomes in neurons.....	17
1.9: Phosphorylation of the Rpt6 subunit of 26S proteasomes.....	19
1.10: Acknowledgments.....	21
Chapter 2: Regulation of the ubiquitin ligase Nedd4-1 at excitatory synapses.....	24
2.1: Introduction.....	25
2.2: Results.....	26
2.3: Conclusions.....	37
2.4: Acknowledgments.....	37

Chapter 3: Synaptic structure and function are altered by the neddylation inhibitor	
MLN4924.....	46
3.1: Introduction.....	47
3.2: Results.....	47
3.3: Conclusions.....	51
3.4: Acknowledgments.....	51
Chapter 4: Role of Rpt6 S120 phosphorylation in synaptic function and plasticity	55
4.1: Introduction.....	56
4.2: Results.....	57
4.3: Conclusions.....	61
4.4: Acknowledgments.....	62
Chapter 5: Conclusions.....	66
5.1: Synaptic activity recruits the E3 ubiquitin ligase Nedd4-1.....	67
5.2: Nedd4-1 regulates homeostatic downscaling of synaptic strength.....	69
5.3: AMPAR ubiquitination as a mechanism for A β -induced synaptic depression.....	70
5.4: Neddylation in excitatory synapse maintenance.....	72
5.5: Proteasome Rpt6 phosphorylation and synaptic plasticity.....	73
5.6: Relationship between S120 phosphorylation and Rpt6 cellular distribution.	75
Chapter 6: Materials and methods.....	77
References.....	88

LIST OF ABBREVIATIONS

A β :	amyloid beta
AD:	Alzheimer's disease
AMPA:	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA:	analysis of variance
APP:	amyloid precursor protein
APV:	DL-2-amino-5-phosphonopentanoic acid (APV)
BAPTA:	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Bic:	bicuculline
BSA:	bovine serum albumin
CA1:	cornu ammonis 1
CA3:	cornu ammonis 3
CaMKII:	calcium/calmodulin-dependent kinase II
CHO:	Chinese hamster ovary
CNS:	central nervous system
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
DG:	dentate gyrus
DIV:	days in vitro
DMSO:	dimethyl sulfoxide
DMEM:	Dulbecco's modified Eagle medium
DUB:	deubiquitinating enzyme
EGFR:	epidermal growth factor receptor
EGTA:	ethylene glycol tetraacetic acid
fEPSP:	field excitatory postsynaptic potential
GABA:	gamma-aminobutyric
GFP:	green fluorescent protein
HA:	hemagglutinin

HBS: HEPES buffered saline
HECT: homologous to the E6-AP carboxyl terminus
HEK: human embryonic kidney
IEI: inter-event interval
KD: knockdown
LTD: long-term depression
LTP: long-term potentiation
mAb: monoclonal antibody
mEPSC: miniature excitatory postsynaptic current
NEDD: neural precursor cell expressed developmentally down-regulated protein
NMDAR: N-methyl-D-aspartate receptor
NR: NMDA receptor
P: postnatal day
pAb: polyclonal antibody
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PKA: protein kinase A
PKC: protein kinase C
PPF: paired pulse facilitation
PSD: postsynaptic density
RING: really interesting new gene
SC: Schaffer collateral
SDS: sodium dodecyl sulfate
TTX: tetrodotoxin
USP8: ubiquitin specific peptidase 8
WT: wild-type

LIST OF FIGURES

Figure 1-1: Ubiquitin-dependent AMPAR trafficking.....	22
Figure 1-2: Process of neddylation and action of the drug MLN4924.....	23
Figure 2-1: AMPAR activation leads to rapid redistribution and accumulation of Nedd4-1 at synapses.....	39
Figure 2-2: Nedd4-1's C2 domain regulates its trafficking and function in Neurons.....	40
Figure 2-3: Synaptic activity levels regulate Nedd4-1.....	41
Figure 2-4: Nedd4-1 is required for homeostatic downscaling.....	42
Figure 2-5: A β -induced synaptic alterations coincide with enhanced AMPAR ubiquitination.....	43
Figure 2-6: A β promotes Nedd4-1 redistribution into dendritic spines and increases Nedd4-1 association with surface proteins.....	44
Figure 2-7: Nedd4-1 is necessary for A β -induced synaptic alterations.....	45
Figure 3-1: Inhibition of neddylation with MLN4924 reduces mEPSC amplitude..	52
Figure 3-2: MLN4924 reduces surface AMPAR expression.....	53
Figure 3-3: Inhibition of neddylation affects dendritic spines.....	54
Figure 4-1: Hippocampal synaptic transmission and long-term potentiation are unaltered in S120 knock-in mice.....	63
Figure 4-2: Fear memory is not impaired in S120 mutant mice.....	64
Figure 4-3: S120 phospho-status does not affect Rpt6 localization in dendrites....	65
Figure 5-1: Involvement of ubiquitin-dependent AMPAR trafficking in synaptic downscaling.....	76

ACKNOWLEDGMENTS

I would like to first thank my advisor, Dr. Gentry Patrick, for his excellent mentorship and support throughout my time in his lab. He consistently serves as a strong advocate for the students in his lab and has always made me feel respected and appreciated. He allowed me a great deal of independence in my research, which has unquestionably improved my abilities as a scholar and a scientist. His relentless positivity, enthusiasm, and kindness made my graduate school experience an incredibly enjoyable one.

I would also like to thank the other members of my thesis committee for the immense amounts of advice and support they have provided me. Dr. Robert Malinow provided me with excellent guidance, equipment, and lab space for my electrophysiology experiments, making Chapter 4 of this dissertation possible. Dr. Brenda Bloodgood has constantly supported and advised me scientifically, professionally, and personally. Dr. Yishi Jin and Dr. Jeff Isaacson have both provided invaluable input on my work at every meeting and have also provided lots of support when I was unsure about my research.

All members of the Patrick laboratory deserve an immense amount of thanks for their scientific help and companionship. Marisa Goo has worked closely with me on several projects and her drive and work ethic is incredible. Toward the end of my research I was fortunate enough to begin working with Frankie Gonzales, who has provided plenty of advice and expertise on biochemistry (and beer). All of the experiments in the lab would be impossible without the technical expertise of Lara Dozier, who consistently generates the most beautiful cultured neurons in all of

neuroscience. All of the other past and present members of the lab have also contributed to a consistently positive atmosphere. Members of the Malinow lab made the research in Chapter 4 possible; I'd specifically like to thank Yvonne Pao for teaching me the art of LTP, and Sage Aronson and Chris Proulx for kindly sharing a rig with me. Stephanie Alfonso also provided plenty of helpful advice on all things related to electrophysiology.

I would also like to thank my friends and classmates for all of their support and good company throughout the good and tough times of graduate school. In particular, my fiancé Tommy Sprague has provided me with endless love, post-ephys backrubs, and scientific advice, and he has served as my primary adventure partner in San Diego and beyond.

Some sections of Chapter 1 contain text prepared by the dissertation author for a recently published review article:

Goo, M.S., Scudder, S.L., Patrick, G.N. "Ubiquitin-dependent trafficking and turnover of glutamate receptors" (Review). 2015. *Frontiers in Molecular Neuroscience*: 8:60.

Marisa Goo was a coauthor on this paper, though all text in this dissertation was written solely by the dissertation author.

Chapter 2 contains research published in the following article:

Scudder, S.L., Goo, M.S., Cartier, A.E., Molteni, A., Schwarz, L.A., Wright, R., Patrick, G.N. "Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8." 2014. *The Journal of Neuroscience*: 34(50): 16637-49.

Marisa Goo provided biochemical data and scientific guidance, Anna Cartier and Alice Molteni provided biochemical data (present in the published paper but not in this dissertation), and Rebecca Wright contributed some electrophysiology data (present in the published paper but not in this dissertation). Part of the research plan originated from Lindsay Schwarz during her time in the lab. Chapter 2 also includes data from the following article:

Rodrigues, E.M., Scudder, S.L., Goo, M.S., Patrick, G.N. “A β -induced synaptic alterations require the E3 ubiquitin ligase Nedd4-1.” 2016. *The Journal of Neuroscience*. In Press.

Elizabeth Rodrigues, a former postdoc in the lab, was the primary author and leader of the research project, and provided all of the biochemistry data. Spine density data was obtained by Marisa Goo.

Chapter 3 contains data from the following article:

Scudder, S.L., Patrick, G.N. “Synaptic structure and function are altered by the neddylation inhibitor MLN4924.” 2015. *Molecular & Cellular Neuroscience*: 65: 52-57.

The dissertation author is solely responsible for the data in this chapter. Dr. Eric Bennett provided helpful reagents and advice for this project.

Chapter 4 is currently being prepared for submission and the final paper will include substantial data from Frankie Gonzales, who will serve as co-first-author on the manuscript. Kristin Howell (a graduate student in Stephan Anagnostaras’s lab) collected all behavioral data from the mutant mice and the generation and basic

characterization of the mice was conducted by Lara Dozier. The tentative citation is as follows:

*Gonzales, F.R., *Scudder, S.L., Howell, K.H., Anagnostaras, S.G., Patrick, G.N.

“Rpt6 S120 mutations do not affect synaptic plasticity and learning.”

This research was supported by a NIH Neuroplasticity of Aging Training Grant (T32 AG00216) and a NSF Graduate Research Fellowship awarded to the dissertation author, and a NIH R01 (NS060847) to Gentry Patrick.

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Scudder, S.L., Goo, M.S., Cartier, A.E., Molteni, A., Schwarz, L.A., Wright, R., Patrick, G.N. “Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8.” 2014. *The Journal of Neuroscience*: 34(50): 16637-49.

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ABSTRACT OF THE DISSERTATION

Activity-dependent regulation of ubiquitination and protein degradation controls
excitatory synaptic transmission and plasticity

by

Samantha Lynn Scudder

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2016

Professor Gentry Patrick, Chair

The connections between neurons in the central nervous system are constantly changing in number and in strength. This process occurs through development and aging, but is also employed to enable learning and memory. Additionally, neurons must be able to constantly sense general activity patterns and make slow adjustments in a homeostatic manner in order for inputs to remain meaningful. One of the ways in which neurons can manage the control of their connections is through manipulations of proteins at synapses. This can be accomplished through control of protein synthesis, trafficking, and degradation. Glutamate receptors such as AMPA receptors as well as a

number of postsynaptic scaffolding proteins are subject to a variety of posttranslational modifications and can be synthesized and degraded in an activity-dependent manner. Recently, it has become clear that multiple steps in degradation pathways can be affected by changes in synaptic activity, and that this regulation can have an immense impact on synaptic strength. However, the exact mechanisms through which degradation systems are regulated has largely remained a mystery. Here, we explore the regulation both of ubiquitination and proteasome-dependent degradation, uncovering novel ways in which synaptic activity interacts with these systems. First, we demonstrate that the activity-dependent ubiquitination of surface AMPA receptors is accomplished through synaptic recruitment of the ubiquitin ligase Nedd4-1, and show that this ligase is critical in homeostatic downscaling of synaptic strength. Additionally, we unveil a role for this ligase in the synaptic depression observed in models of Alzheimer's disease, illustrating that regulation of Nedd4-1 can go awry. Next, we demonstrate that control of excitatory synapses can also be accomplished with the conjugation of the small ubiquitin-like molecule NEDD8, known to regulate the activity of a number of ubiquitin ligases. Finally, we explore the role of CaMKII-dependent phosphorylation of the Rpt6 subunit of 26S proteasomes in plasticity and learning. These studies add to our knowledge of how protein degradation can be regulated by activity and how this regulation can impact synaptic plasticity.

Chapter 1:

Introduction

The nervous system has the immense capability for growth and change due to the ability of connections to be modified. Learning and memory are enabled in the brain by the formation of new synapses and the alterations of existing ones, termed synaptic plasticity. At excitatory synapses, one of the main mechanisms of synaptic plasticity relies on the addition, removal, and relocation of surface glutamate receptors at the postsynaptic membrane, which allows the strength of that connection to be flexible. This chapter will discuss the main types of plasticity at glutamatergic synapses, the posttranslational modifications that support them, and the emerging role for protein degradation systems in allowing for such plasticity.

1.1: Types of synaptic plasticity in the central nervous system

The mammalian brain consists of a number of distinct anatomical regions and neuronal cell types which participate in a variety of forms of plasticity. For the majority of this dissertation, we will focus on plasticity that occurs within hippocampal neurons, specifically the glutamatergic pyramidal neurons in this region. The hippocampus is a highly plastic circuit that is responsible for the formation of episodic and declarative memories. This region is typically organized into three major regions that participate in a specific connection pattern: the dentate gyrus (DG) projects to the CA3, and the pyramidal neurons in this region project to a region called CA1. These CA3-CA1 projections are also called Schaffer collaterals and are likely the most highly studied projections in the brain due to the high degree of plasticity that can be evoked at the synapses onto CA1 pyramidal neurons. The forms of synaptic plasticity described in this chapter have all been observed at these synapses to some

degree, though several of them can also operate in other brain regions, such as cortical areas.

Neurons can participate in a number of different types of synaptic plasticity, allowing for both strengthening and weakening across several different time scales. The most well-studied paradigms are long-term potentiation (LTP) and long-term depression (LTD), in which a shift of activity at the synapse leads to long-lasting changes to the strength of that synapse, in a positive feedback manner (Huganir & Nicoll, 2013; Anggono & Huganir, 2012). Specifically, a burst of high-frequency activity at an input to a hippocampal neuron causes that specific connection to be strengthened, supported in great part by the addition and stabilization of glutamate receptors at the activated synapses (Andersen et al., 1977; Hayashi et al., 2000; Kessels & Malinow, 2009). It is believed that this form of hippocampal plasticity provides the mechanism for the formation of new episodic and declarative memories (Morris et al., 1986; Bliss & Collingridge, 1993; Whitlock et al., 2006). The converse is long-term depression, in which a low-frequency activity pattern leads to the selective weakening of synapses through removal of surface receptors (Dudek & Bear, 1992). These mechanisms are both referred to as Hebbian plasticity and have been the focus of the bulk of studies on synaptic plasticity. In both cases, the early phase of plasticity is supported mainly by alterations to postsynaptic receptor abundance, but long-term maintenance of these changes requires structural remodeling of synapses, a process which requires new protein synthesis as well as protein degradation (Sutton & Schuman, 2005; Bramham, 2008).

While Hebbian plasticity largely deals with acute changes in activity at synapses, chronic changes in activity can also affect neuronal connections. In this case, synapses can experience a form of negative feedback regulation termed homeostatic synaptic plasticity, wherein a neural network attempts to maintain stable and meaningful connections in the face of shifts in overall activity (Pozo & Goda, 2010). While this can be accomplished through a number of network-level changes to adjust excitatory and inhibitory drive, each neuron also has the capacity to regulate its own inputs to stabilize its firing rate (Wang et al., 2012). Synaptic scaling is the process by which a neuron strengthens or weakens its synapses, often through the trafficking of glutamate receptors, in response to a chronic change in activity (Turrigiano, 2008; Hugarir & Nicoll, 2013). In cultured neurons, the drug tetrodotoxin (TTX) is utilized to silence neurons and trigger upscaling, while the inhibitory synapse blocker bicuculline can be used to globally increase activity and trigger the scaling down of excitatory synapses (Turrigiano et al., 1998; Siddoway et al., 2013). While the functional relevance of synaptic scaling is still under considerable investigation, it has been shown to play a critical role in activity-dependent changes in visual areas during development (Keck et al., 2013; Hengen et al., 2013) and is also theorized to function during sleep to re-calibrate connections (Tononi & Cirelli, 2003; Wang et al., 2011; Grosmark et al., 2012).

1.2: Posttranslational modifications of glutamate receptors

Posttranslational modifications such as phosphorylation (the attachment of a phosphate group to a serine, threonine, or tyrosine by a kinase) have long been known

to control the trafficking of surface glutamate receptors, and such modifications can occur specifically in response to synaptic cues, such as changes in activity or the presence of modulatory neurotransmitters (Huganir & Nicoll, 2013; Lussier et al., 2015). While all three glutamate receptor subtypes (AMPA, NMDA, and kainate) can be modified and trafficked, AMPA receptors (AMPA receptors) mediate the majority of excitatory transmission and thus the trafficking of these receptors is crucial for synaptic plasticity (Shepherd & Huganir, 2007; Lu & Roche, 2012). These tetrameric receptors can be composed of four different subunits, termed GluA1-4, with each subunit being subject to specific types of regulation (Shi et al., 2001; Traynelis et al., 2010). When a phosphate group is added to serine 880 on the C-terminal intracellular tail of GluA2, for example, it serves as a signal for endocytosis (Chung et al., 2000; Seidenman et al., 2003), while phosphorylation of serine 845 on GluA1 seems to promote or maintain its presence at the synapse and enhance the receptor's function (Roche et al., 1996; Lee et al., 2003). Modifications at these sites, and many others, occur in response to changes in activity, such as stimulation paradigms that can evoke LTP or LTD.

The various kinases and phosphatases that target AMPARs must thus be subject to regulation by activity, via signaling mechanisms such as calcium influx. Calcium/calmodulin-dependent kinase II (CaMKII) is a particularly prolific kinase which is activated following synaptic activity and serves to promote plasticity using several mechanisms (Lee et al., 2009; Lisman et al., 2012). In addition to the transient activity increases this kinase undergoes, CaMKII can also be dynamically recruited to dendritic spines (Shen & Meyer, 1999; Merrill et al., 2005). Protein Kinase A (PKA)

also plays an important role in regulating AMPAR trafficking and is also subject to activity-dependent spine recruitment and activation (Zhong et al., 2009; Esteban et al., 2003). While much of this research has focused on proteins involved in LTP and LTD, in recent years there has also been a push to determine the types of signaling pathways that control synaptic scaling and thus allow for the homeostatic control of neuronal activity on a long time scale rather than in response to acute perturbations.

Though it was not initially appreciated as a potential regulator of synaptic strength, it is now known that the posttranslational addition of the small protein ubiquitin to the lysine residues of a variety of synaptic proteins can serve to regulate synaptic transmission and plasticity. The addition of ubiquitin to a substrate involves a series of steps that require an activating enzyme (E1), a conjugating enzyme (E2), and eventually a ligase (E3) that confers target specificity (Hershko & Ciechanover, 1998). Traditionally, the ubiquitination of proteins was considered to be a quality control mechanism that broadly regulates the turnover of proteins in all cell types, causing the targeted proteins to be sent to lysosomes or proteasomes for degradation. While this continues to be true in neurons, ubiquitin also plays a complex role in regulating the localization and abundance of synaptic proteins such as glutamate receptors (Yamada et al., 2013). Importantly, this is not merely a constitutive process; AMPA receptors can become ubiquitinated in response to changes in receptor activation, and this results in removal from the synapse and eventual degradation (Fig. 1-1; Schwarz et al., 2010; Lin et al., 2011; Lussier et al., 2011). Thus, ubiquitin serves as a functional modification in the control of synaptic strength.

1.3: Regulation of AMPAR ubiquitination

The first study of mammalian AMPAR ubiquitination determined that direct activation of receptors with the agonist AMPA causes robust ubiquitination of the GluA1 subunit (Schwarz et al., 2010), a finding which has been confirmed in recent studies (Widagdo et al., 2015). Interestingly, another group found that AMPA promotes ubiquitination of the GluA2 subunit rather than GluA1 (Lussier et al., 2011). However, a recent report indicates that AMPA induces the ubiquitination of all four AMPAR subunits (Widagdo et al., 2015). Regardless of this discrepancy, all available data support the conclusion that direct activation of AMPARs with agonists promotes their ubiquitination and internalization. This ligand-induced effect requires calcium entry, provided mainly through voltage-gated calcium channels while NMDA receptor (NMDAR) signaling appears unnecessary (Schwarz et al., 2010; Lussier et al., 2011; Widagdo et al., 2015).

In addition to bath application of receptor agonist, many groups have used alternate methods to examine activity-induced AMPAR ubiquitination. The GABA_A receptor antagonist bicuculline is commonly used to globally raise activity in cultured neurons, and when applied to neurons for a prolonged amount of time can induce a negative feedback process termed synaptic scaling (Turrigiano et al., 1998; Siddoway et al., 2014). Bicuculline treatments have been shown to promote AMPAR ubiquitination after short-term and long-term treatments (Lussier et al., 2011; Widagdo et al., 2015). However, unlike the AMPA-induced scenario, this form of receptor modification appears to require NMDAR signaling, which suggests there may be slight differences in the pathways that involve ubiquitin conjugation. Application of

an agonist activates both synaptic and extrasynaptic receptors while bicuculline should only activate synaptic AMPARs, and this difference may activate different cellular pathways and perhaps even lead to different receptor fates. Alternatively, these two scenarios may simply differ in the source of calcium; NMDARs could provide the calcium influx during bicuculline treatments while AMPA treatments instead rely on calcium influx through voltage-gated calcium channels and calcium-permeable AMPARs.

The specific ligase responsible for bicuculline-induced ubiquitination is debated; short treatments induce AMPAR ubiquitination that requires the ligase RNF167 (Lussier et al., 2012), but prior to our work, ubiquitination under long-term activity changes had not been examined. The E3 ligase complex APC^{Cdh1} also appears to become engaged upon long-term bicuculline treatment, as loss of this protein prevents bicuculline's effects on synaptic strength, though it is unclear whether this is due to direct targeting of AMPARs (Fu et al., 2011). RNF167 may handle short-term regulation of surface AMPARs while other ligases act on a longer time scale to homeostatically control synaptic strength (Fig. 1-1).

In addition to pharmacological manipulations, AMPAR ubiquitination has been studied using various other techniques. Using light-gated glutamate receptors to activate a subset of cultured neurons, Hou et al. demonstrated that synapses receiving 30 min of prolonged activity reduced their total and surface GluA1 and also experienced a site-dependent increase in polyubiquitin conjugates and the ligase Nedd4-1 (Hou et al., 2011). These data suggest that homeostatic scaling via ubiquitin-dependent pathways can occur on a single synapse level. To date, only a few groups

have examined the role of receptor ubiquitination *in vivo* (Yuen et al., 2012; Atkin et al., 2015). Yuen et al. found that repeatedly exposing rats to stress leads to ubiquitination of GluA1 and the NMDAR subunit NR1 in the prefrontal cortex by Nedd4-1 and Fbx2, respectively, and that this results in reduced levels of these receptors and reduced glutamatergic transmission, which may underlie the stress-induced cognitive deficits observed.

1.4: Function of ubiquitination in AMPAR trafficking

The field has converged on the idea that ubiquitination plays a critical role in regulating the abundance and localization of AMPARs in neurons. However, the exact role that ubiquitin conjugation plays remains debated. Direct conjugation to AMPAR subunits at the cell surface may function as a signal for internalization by triggering the assembly of endocytic machinery. Alternatively, the internalization process may occur prior to conjugation, with ubiquitination instead serving to direct endocytosed receptors toward a fate of degradation and prevent them from recycling to the surface.

In *C. elegans* it was first observed that the abundance of GLR-1, the *C. elegans* non-NMDA type glutamate receptor, is regulated by ubiquitin (Burbea et al., 2002; Juo and Kaplan, 2004). GLR-1 was found to be ubiquitinated *in vivo*, and mutations in GLR-1 which block ubiquitination increase the abundance of the receptor at synapses and alter locomotion behavior in a manner consistent with increased synaptic strength. In this system, overexpression of ubiquitin caused a decrease in GLR-1 abundance, and mutations in *unc-11*, which encodes the clathrin adaptin protein AP180, blocked the effect. Additionally, ubiquitin-conjugated GLR-1 accumulated in neurons lacking

functional AP180. While the authors acknowledged that it is possible that ubiquitination is occurring in endosomes to control degradation, their data strongly supports a model where ubiquitination of GLR-1 occurs at the surface, prior to internalization through clathrin-mediated endocytosis (Burbea et al., 2002).

Three of the first papers to study mammalian AMPAR ubiquitination argued in favor of ubiquitination at the postsynaptic membrane (Schwarz et al., 2010; Fu et al., 2011; Lin et al., 2011). In these studies, blocking ubiquitination by mutating the relevant GluA1 lysines (GluA1-4KR) or knocking down the E3 ligase responsible (Nedd4-1 or Cdh1, activator of APC) prevented the detection of internalized GluA1 during a stimulation-induced internalization assay. Thus, the authors concluded that ubiquitination of AMPAR subunits is a necessary step in the internalization of stimulated receptors. However, an alternate explanation could be that surface receptors are indeed internalized in these conditions, but upon failure of ubiquitination in a nascent endosomal vesicle, receptors are recycled back to the membrane during the time-frame of the internalization assay. In that case, the lack of a sorting signal by ubiquitin would cause the same observed effect as the lack of internalization. However, in agreement with the aforementioned studies, a recent paper identified a role for the endocytic adaptor protein Eps15, which is known to be critical in supporting the internalization of epidermal growth factor receptor (Goh and Sorkin, 2013), in ubiquitin-dependent receptor trafficking (Lin and Man, 2014). The authors found that levels of Eps15 affected surface expression of GluA1 through ubiquitin-dependent interactions with this subunit and also demonstrated that clathrin-mediated endocytosis is necessary for the ubiquitin-induced enhancement in receptor

internalization (Lin and Man, 2014). Since Eps15 is involved in the recruitment of endocytic machinery at the surface, the authors conclude that this ubiquitin-mediated interaction is occurring prior to AMPAR internalization.

There have been studies of AMPAR ubiquitination which concluded that ubiquitination is not necessary for the internalization step of this pathway (Lussier et al., 2011; Widagdo et al., 2015). In investigating activity-induced GluA2 ubiquitination, Lussier et al. utilized dynasore to block dynamin-mediated endocytosis and sucrose to prevent formation of clathrin-coated pits and observed that these manipulations prevent the detection of bicuculline-induced ubiquitination of GluA2 (Lussier et al., 2011). This supports a model where ubiquitin conjugation occurs after internalization to control receptor sorting. Similarly, a recent paper observed that inhibition of dynamin-mediated endocytosis with dynasore abolishes bicuculline- or AMPA-induced ubiquitination of all four AMPAR subunits (Widagdo et al., 2015). Curiously, this study also utilized GluA1 mutants that cannot be ubiquitinated but found that these lysine mutations did not prevent agonist-induced internalization, in contrast to the previously described papers (Schwarz et al., 2010; Lin et al., 2011). Instead, the lysine mutations reduced the amount of internalized GluA1 that co-localized with LAMP-1 positive late endosome/lysosomes and allowed more GluA1 to return to the surface. As a result, the authors conclude that ubiquitination occurs after receptors have been internalized, likely in early endosomes.

While the role of ubiquitination in regulating AMPARs has only been explored fairly recently, extensive work has been done to identify the role of ubiquitin in controlling surface proteins in non-neuronal cells. Epidermal growth factor receptor

(EGFR) has been the subject of numerous studies, as its ligand-induced removal from the cell surface is regulated by the E3 ubiquitin ligase c-Cbl (Goh and Sorkin, 2013). Though considerable debate continues to exist surrounding the various internalization pathways, ample evidence has shown that direct ubiquitination of EGFR by c-Cbl can serve as a signal for the assembly of clathrin-mediated endocytic machinery (including Eps15) and subsequent endocytosis (Stang, 2000; de Melker et al., 2001). However, EGFR can also be internalized through a non-ubiquitin-dependent pathway, and ubiquitination can instead occur while EGFR is located in endosomes, where it serves as a signal for degradation (Levkowitz et al., 1998; Huang et al., 2007; Goh and Sorkin, 2013). Thus, it is reasonable that AMPAR internalization could occur through multiple pathways that involve ubiquitin, and that ubiquitination can occur on the surface to signal internalization or at early or late endosomes to control receptor fate. The exact conditions (i.e., type and intensity of neuronal stimulation) could determine which pathway surface AMPARs engage in. Since there appear to be a few distinct E3 ligases that can target AMPAR subunits (Fig. 1-1), these ligases may engage the receptors at different points in the internalization process. Additionally, since AMPARs can be composed of four different subunits, the exact composition of surface receptors may also be a factor.

1.5: AMPAR loss in Alzheimer's disease

Neurons must have mechanisms for reducing synaptic strength in order to maintain stable connections within a dynamic network. These mechanisms are thus critical for the health of the nervous system. However, excessive endocytosis can have

detrimental effects on neuronal health and brain function. Alzheimer's disease is a progressive neurodegenerative disorder that is characterized by certain pathological features, such as amyloid beta plaques and neurofibrillary tangles, as well as severe cognitive decline. While widespread neuronal loss is seen in the late stages of this disease, it is believed that the earliest changes are at individual synapses (Selkoe, 2002). A slow weakening of excitatory connections could be the first step in the decline of neuronal function.

Discovering the exact mechanism of synaptic weakening is of great interest, as this would both clarify disease pathogenesis and potentially open the door to novel pharmacological interventions. As studying such a mechanism in humans is quite challenging, many researchers utilize animal models and cell culture models to mimic aspects of the disease, often using application of amyloid beta (A β) oligomers or overexpression of the precursor protein APP to induce synaptic weakening and cognitive decline (Hardy & Selkoe, 2002; Almeida et al., 2005). It has been observed in both animal models of Alzheimer's and human patients that excitatory synapses exhibit a loss of surface and total AMPARs (Carter et al., 2004; Hsieh et al., 2006). This loss causes reduced excitatory transmission and plasticity, particularly in temporal lobe regions, which are known to be heavily involved in the generation of long-term memory (Chang et al., 2006; Shankar et al., 2008). It is theorized that an overabundance of AMPAR endocytosis is to blame for this; internalization machinery may be overactive, leading to a pathological weakening of synapses (Hsieh et al., 2006). One potential mechanism appears to involve protein kinase C, as inhibition of this kinase prevents the synaptic deficits induced by amyloid beta treatments (Liu et

al., 2010; Alfonso et al., 2014). However, the exact details of A β -induced AMPAR internalization remain unclear.

Since ubiquitin-dependent trafficking of AMPARs serves as a negative regulator of synaptic strength, it could also be a mechanism that goes awry in Alzheimer's disease. If the protein levels, cellular distribution, or activity levels of the ubiquitin ligase Nedd4-1 were altered, neurons could begin to exhibit pathologically weakened synapses. Interestingly, it was recently reported that total levels of Nedd4-1 are increased in brain samples from Alzheimer's patients and are also increased in cultured neurons in response to A β oligomer treatments (Kwak et al., 2012). This suggests that A β may be somehow affecting Nedd4-1, possibly utilizing it to induce changes at excitatory synapses. In Chapter 2, we detail our work in exploring a role for Nedd4-1 in A β -induced synaptic depression.

1.6: Role of the ubiquitin-like molecule NEDD8 in the nervous system

As previously described, ubiquitination controls the targeting of specific substrates to 26S proteasomes and lysosomes for degradation and can also mediate subcellular distribution and function. Substrate specificity is accomplished by E3 ubiquitin ligases, which are responsible for the final step in the ubiquitination process. These enzymes are divided into a number of different families based on their structure and function, and their activity can be regulated through posttranslational modifications.

The conjugation of the ubiquitin-like protein NEDD8, termed neddylation, has been identified as a regulatory mechanism for a number of E3 ligases and components

of E3 complexes (Rabut and Peter, 2008). Notably, all members of the cullin family of proteins, which comprise multi-subunit E3 ligase complexes, are known to be neddylated (Jones et al., 2008; Osaka et al., 1998). While the exact biological effects of neddylation are still unclear, ample evidence suggests that the conjugation of NEDD8 to cullin proteins stimulates the ligase activity of cullin-based E3 complexes (Bennett et al., 2010; Podust et al., 2000; Read et al., 2000) and parkin (Um et al., 2012). Members of other E3 ligase families have also been shown to be substrates for neddylation, including the RING finger ubiquitin ligase Mdm2 and the HECT ligase Smurf1 (Xie et al., 2014; Xirodimas et al., 2004). This suggests that neddylation plays a critical role in regulating ubiquitination, potentially by orchestrating the activity of a number of different ligases. Interestingly, many ubiquitin ligases known to be neddylated have also been demonstrated to regulate neuronal function and synaptic strength through the ubiquitination of synaptic proteins (Chen and Matesic, 2007; Cheng et al., 2011; Choo et al., 2012; Colledge et al., 2003; Zhu et al., 2005; Chen et al., 2012). However, little work has been done to directly explore the role of neddylation in neurons.

The recent generation of the NEDD8 activating enzyme (NAE) inhibitor MLN4924 has enabled a number of studies exploring the roles of neddylation in various cell types (Fig. 1-2) (Bennett et al., 2010; Gu et al., 2014; Soucy et al., 2009). Though initially created as a potential tumor inhibitor, this drug allows for the pharmacological exploration of NEDD8 function. In Chapter 3 we describe our use of this compound to demonstrate that inhibition of neddylation in cultured hippocampal rat neurons affects synaptic strength and dendritic spine morphology. Our results

provide evidence that neddylation plays a role in the maintenance of excitatory synapses.

1.7: Fate of ubiquitinated proteins

While the previously described work focuses only on the effects of removing AMPARs from the synapse and thus reducing synaptic strength, it is also intriguing to explore what happens to synaptic proteins once they have been ubiquitinated. In all eukaryotic cell types, ubiquitination can lead to a number of fates for the target protein, including degradation by lysosomes or proteasomes (Hershko & Ciechanover, 1998; Clague & Urbé, 2010). Lysosomes can degrade large cytosolic components via autophagy (Shintani et al., 2004) and can also degrade surface proteins (such as receptors) via the endosomal sorting complexes required for transport (ESCRT) pathway (Hurley, 2008; Henne et al., 2011). Internalized, ubiquitinated AMPARs can be routed through this pathway, resulting in their eventual degradation by lysosomes if they are not spared by deubiquitination (Schwarz et al., 2010; Widagdo et al., 2015). Mono- or short chain- ubiquitination appears to be the signal for this lysosomal pathway to be utilized. Thus, lysosomes likely play a critical role in supporting synaptic plasticity, though the regulation of their localization and function has yet to be explored in neurons.

The other major degradation site in eukaryotic cells is the 26S proteasome, which is a large multi-subunit complex composed of a 20S core subunit containing proteolytic components and two 19S cap complexes that serve as critical regulators of proteasome function (Bedford et al., 2010). These cap particles consist of multiple

ATPase and non-ATPase subunits, which work together to recognize polyubiquitinated substrates and route them into the proteolytic core for degradation (Rubin et al., 1998; Navon & Goldberg, 2001). These subunits can undergo posttranslational modifications that control their conformation and function, which can affect the overall function of the proteasome (Zhang et al., 2003; Bardag-Gorce et al., 2004). Additionally, these subunits can have interactions with other proteins, and thus can regulate the function and the cellular distribution of proteasomes throughout the cell (Gorbea et al., 2004; Tai et al., 2010).

1.8: Proteasomes in neurons

Degradation of synaptic proteins by proteasomes is known to be an important factor in the control of synaptic strength. Long-term changes in synaptic activity, such as those evoked by TTX and bicuculline, induce both the synthesis and degradation of a variety of synaptic proteins (Ehlers, 2003; Piccoli et al., 2007; Pak & Sheng, 2003). More acute manipulations, such as brief activation of glutamate receptors, also induce proteasome-dependent degradation of postsynaptic proteins such as PSD-95, GRIP1, and in some cases AMPARs (Colledge et al., 2003; Guo & Wang, 2007; Hou et al., 2011).

Manipulations of proteasome activity appear to affect a number of types of synaptic plasticity. For example, a number of groups have reported that proteasome inhibition affects the induction and maintenance of LTP, with an apparent enhancement in initial induction and an impairment in late phase LTP maintenance (Karpova et al., 2006; Fonseca et al., 2006; Dong et al., 2008). LTD also appears to

rely on proteasomal degradation, as application of inhibitors greatly diminish depression (Colledge et al., 2003). Long-term inhibition of proteasome activity detrimentally affects cell health, which makes the study of homeostatic plasticity in this context difficult, but preventing the turnover of certain proteins by proteasome-dependent degradation has been shown to impair bidirectional scaling (Shin et al., 2012). While the exact mechanisms of these effects have not been fully characterized, these studies suggest that the control of protein turnover is critical in synaptic plasticity, just as has been shown for regulation of protein synthesis. In addition to the synaptic proteins that are directly degraded by proteasomes during plasticity, it has also been demonstrated that proteasomes can degrade both positive and negative regulators of plasticity, such as transcription inhibitors or enhancers, which would allow them to play an extremely complex role in orchestrating both short-term and long-term plasticity (Dong et al., 2014).

In line with the demonstrated role in synaptic plasticity, proteasomes also appear to be critical for certain types of learning and memory. Infusions of proteasome inhibitors into invertebrate nervous systems have negative effects on certain learning paradigms (Sol Fustiñana et al., 2014). In rodents, infusions of inhibitors into the hippocampus impair memory formation in a water maze task (Artinian et al., 2008) and an inhibitory avoidance task (Lopez-Salon et al., 2001), and reconsolidation in a fear conditioning task (Lee et al., 2008). Similarly, infusions into the amygdala impair acquisition of fear conditioning (Jarome et al., 2011). These studies point to a critical role of proteasome-dependent degradation in learning and memory, though they do not

reveal the precise mechanisms through which proteasomes allow plasticity and learning to occur.

Proteasomes are present throughout neurons, and have been observed throughout dendritic arbors and even within individual dendritic spines. This suggests that protein degradation can occur locally, which potentially gives the neuron greater control over the abundance of synaptic proteins at highly specific points throughout the cell. Recent work has determined that these dendritic proteasomes are not fixed to specific locations and are instead able to traffic in and out of spines in response to synaptic activity (Bingol & Schuman, 2006; Shen et al., 2007). The current theory for how this translocation works involves the following steps: activation of NMDA receptors through heightened synaptic activity, influx of calcium through these receptors, activation of calcium/calmodulin-dependent kinase II alpha (CaMKII α), tethering of CaMKII α to proteasomes, and then the translocation and sequestration of proteasomes within the spine (Bingol et al., 2010).

1.9: Phosphorylation of the Rpt6 subunit of 26S proteasomes

The Rpt6 subunit of the 19S regulatory cap particle can undergo phosphorylation at serine 120. CaMKII appears to be largely responsible for this phosphorylation, although protein kinase A (PKA) has also been shown to target this site (Zhang et al., 2007; Djakovic et al., 2009). However, the exact regulation of this serine and the effect it has on proteasome function and localization is highly debated. Evidence points to S120 phosphorylation as a regulator of proteasome proteolytic activity, with the phosphorylated state displaying higher activity (Zhang et al., 2007;

Djakovic et al., 2009). With regards to proteasome localization within neurons, it has been reported that the kinase activity of CaMKII α is in fact dispensable for recruitment to dendritic spines, suggesting instead that it is merely interactions between CaMKII α and proteasomes that allows them to be tethered at the synapse (Bingol et al., 2010). However, mutations to this site affect the detergent-resistant extraction of proteasomes, which could mean that this site regulates interactions with scaffolding proteins in dendrites (Djakovic et al., 2012).

Since S120 phosphorylation is observed in response to activity, it suggests that this site may be a part of a plasticity mechanism. Indeed, when phospho-dead (serine to alanine, S120A) or phospho-mimetic (serine to aspartic acid, S120D) variants of Rpt6 are introduced to cultured neurons, synaptic transmission and homeostatic scaling are altered in a bidirectional manner (Djakovic et al., 2012). Additionally, activity-induced dendritic spine outgrowth is affected by S120 mutations, pointing to a role for this site in regulating structural plasticity (Hamilton et al., 2012). The role of this phosphorylation site in an intact animal has only been explored in one study thus far, which demonstrated that Rpt6 becomes phosphorylated and proteasome activity increases in the amygdala of rats following a fear conditioning behavioral paradigm (Jarome et al., 2013). While inhibition of CaMKII α in vivo prevented the effects on proteasomes and interfered with memory formation, it is still unclear whether the S120 site itself is playing a mechanistic role in plasticity and learning.

Despite the evidence for a potential role of Rpt6 phosphorylation in synaptic function and plasticity provided by cultured neuron studies, it is still unclear what the importance of this particular site is in an intact circuit or organism. Additionally, many

previous studies utilized over-expression rather than replacement of endogenous Rpt6, which could greatly alter proteasome composition, function, and abundance.

Manipulations of the responsible kinases, CaMKII α and PKA, do affect plasticity and learning, but this provides little information as these kinases have a number of synaptic targets. In order to study the role of S120 phosphorylation in learning and memory and types of plasticity that support such processes, the endogenous protein must be modified in an animal model. Thus, we generated knock-in mice expressing the S120A and S120D mutations. Chapter 4 focuses on our characterization of the synaptic function and plasticity in these mice.

1.10: Acknowledgments

Some sections of Chapter 1 contain text prepared by the dissertation author for a recently published review article:

Goo, M.S., Scudder, S.L., Patrick, G.N. “Ubiquitin-dependent trafficking and turnover of glutamate receptors” (Review). 2015. *Frontiers in Molecular Neuroscience*: 8:60.

Marisa Goo was a coauthor on this paper, though all text in this dissertation was written solely by the dissertation author.

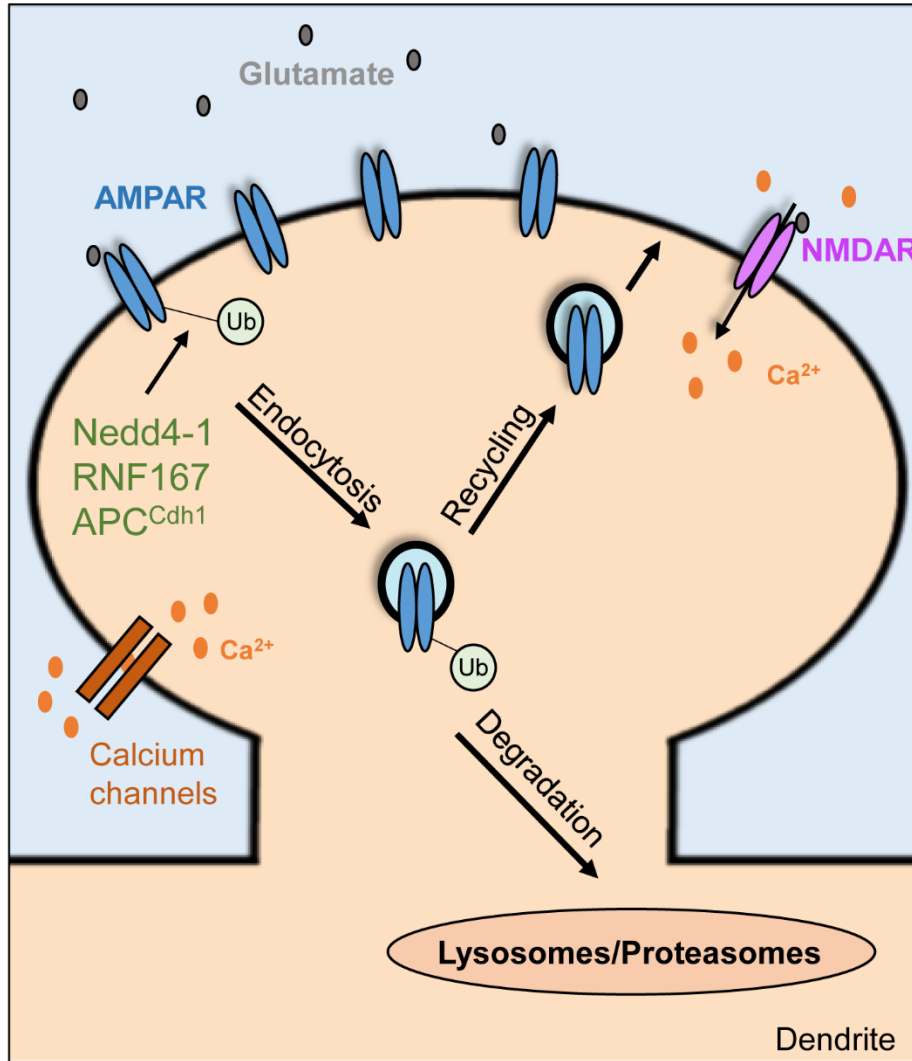


Figure 1-1: Ubiquitin-dependent AMPAR trafficking. At excitatory synapses, activation of surface AMPARs through glutamate or the agonist AMPA can induce the intracellular ubiquitination and internalization of receptors, which can eventually lead to degradation by lysosomes or proteasomes. Three different E3 ubiquitin ligases are known to ubiquitinate AMPAR subunits: Nedd4-1, RNF167, and APC^{Cdh1}. Calcium influx through calcium channels or NMDARs is necessary for AMPAR ubiquitination and internalization.

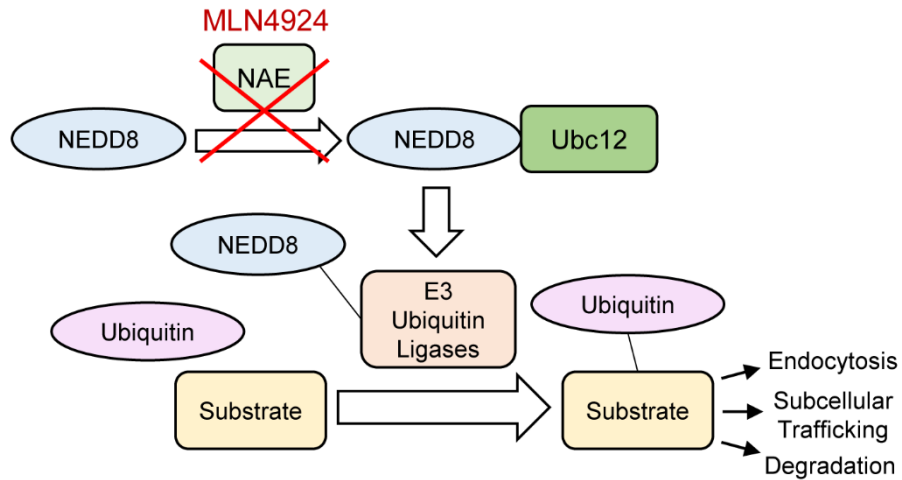


Figure 1-2: Process of neddylation and action of the drug MLN4924. Under normal conditions, NEDD8 activating enzyme (NAE) permits NEDD8 to be conjugated to its intermediate E2 enzyme (Ubc12) and then conjugated to its target substrates, which include a number of E3 ubiquitin ligases. The stabilizing and activating effects of NEDD8 conjugation allow the E3 ligases to conjugate ubiquitin to their specific protein substrates, which serves as a signal for surface protein endocytosis, trafficking between subcellular compartments, or degradation via proteasomes or lysosomes. MLN4924 inhibits this regulatory pathway through direct inhibition of NAE.

Chapter 2:

Regulation of the ubiquitin ligase

Nedd4-1 at excitatory synapses

2.1: Introduction

Despite the large body of work that has been completed over the last decade or so, there still remain a number of open questions regarding the role of ubiquitination in neurons. Specifically, how are the ubiquitin ligases that target AMPARs being regulated by synaptic activity? What sorts of regulatory mechanisms to neurons employ to control the activity and localization of E3 ligases? Are there diseases in which dysfunction in these pathways can contribute to synaptic defects, and how could this occur? In this chapter, we thoroughly explore these questions.

We focus on Nedd4-1, a ligase previously shown by our lab to target AMPARs (Schwarz et al., 2010). Previous work demonstrated that the GluA1 subunit of AMPARs becomes subject to short-chain and mono-ubiquitination in response to activation of receptors by the agonist AMPA. This ubiquitination relies on the presence of the E3 ligase Nedd4-1, and altering the levels of Nedd4-1 via overexpression leads to a reduction in synaptic strength and surface GluA1 expression. This suggests that Nedd4-1 serves as a negative regulator of AMPAR abundance at synapses and that its ability to do so is dependent on activation of receptors. Additionally, it also positions Nedd4-1 as a good candidate to operate in homeostatic plasticity. Since synaptic down-scaling involves the removal of synaptic AMPA receptors and is triggered by an increase in activity levels, Nedd4-1 would be an intriguing candidate to mediate the connection between changes in activity levels and changes in synaptic strength.

Here, we demonstrate that this ligase undergoes activity-dependent redistribution to excitatory synapses. We also provide evidence that this ligase plays a

crucial role in the homeostatic process of synaptic downscaling. Additionally, we explore the role of Nedd4-1 in a cell-culture model of Alzheimer's disease and identify it as a potential mediator of the synaptic defects observed in this disease. The work presented in Chapter 2 provides strong evidence that the regulation of ubiquitination in neurons is crucial in allowing these cells to dynamically control their synapses.

2.2: Results

Nedd4-1 is rapidly redistributed to synapses in response to AMPAR activation

It has been previously shown that the application of AMPA or NMDA to hippocampal neurons promotes the internalization of (AMPA)Rs (Ehlers, 2000; Lin et al., 2000; Lee et al., 2004). However, we and others recently showed that these stimuli differ in their ability to induce the ubiquitination of AMPARs (Schwarz et al., 2010; Lussier et al., 2011). We predicted that Nedd4-1, a ubiquitin ligase which targets AMPARs, might be itself differentially regulated by specific synaptic cues. Therefore, we hypothesized that rapid regulation of Nedd4-1 could be achieved by control of its localization in neurons. In order to clearly visualize the cellular distribution of Nedd4-1 under various conditions, we infected dissociated hippocampal neurons with a Sindbis virus expressing cell-filling GFP and an HA-tagged Nedd4-1. As previously described, this tagged Nedd4-1 displays a similar cellular distribution to endogenous Nedd4-1 (Schwarz et al., 2010). We then asked whether synaptic activity induces any change to the distribution of Nedd4-1 by bath-applying glutamate and glycine to neurons expressing HA-Nedd4-1 (Fig. 2-1). We observed a robust change in

distribution of Nedd4-1 in the treated condition compared to untreated neurons, quantified through a custom macro (see Materials and Methods). Specifically, the distribution of HA-Nedd4-1 changed from a diffuse pattern throughout dendrites and soma into a punctate pattern with tightly accumulated signal in dendritic spines ($p < 0.001$; Fig. 2-1A, C). Moreover, we found extensive co-localization of HA-Nedd4-1 with the postsynaptic density protein PSD-95, and juxtaposition to the presynaptic protein synapsin I (Fig. 2-1H, I), indicating a recruitment to synapses. Since glutamate activates both AMPA and NMDA receptors at synapses, we next tested the ability of AMPA and NMDA to induce these changes. AMPA induced a robust redistribution of Nedd4-1 while NMDA produced no effect ($p < 0.001$, $p = 0.44$, respectively; Fig. 2-1B, C), indicating that activation of surface AMPARs is necessary for the recruitment of Nedd4-1.

We next explored the properties of this activity-induced redistribution. Nedd4-1 was observed to relocalize rapidly, with significant changes apparent after two minutes of AMPA treatment and a more robust effect at five and ten minutes ($p < 0.01$, $p < 0.001$, respectively; Fig. 2-1D). Additionally, we found that HA-Nedd4-1 remains localized to synapses following wash-out of AMPA, with significant redistribution evident for at least 20 minutes post-washout ($p < 0.001$; Fig. 2-1E). Since previous studies indicated that the AMPA-induced ubiquitination of AMPARs is calcium-dependent (Schwarz et al., 2010; Lussier et al., 2011), we next asked whether the rapid redistribution of Nedd4-1 requires calcium as well. AMPA treatments following a pre-treatment with the calcium buffer EGTA induced a significantly smaller redistribution (two-way ANOVA, interaction: $F(1, 209) = 13.59$, $p < 0.001$),

though the effect was not completely abolished (AMPA vs. EGTA+AMPA: $p < 0.01$; Fig. 2-1F). Interestingly, raising intracellular calcium levels by ionomycin treatment produced no changes to Nedd4-1's localization, indicating that calcium alone is not sufficient to recruit Nedd4-1 to synapses. We hypothesized that redistribution requires both a rise in calcium and ligand binding at AMPA receptors. To test this, we applied the AMPAR antagonist CNQX to serve as a non-activating ligand and ionomycin to raise intracellular calcium. As predicted, application of both CNQX and ionomycin was sufficient to promote significant redistribution ($p < 0.001$) of Nedd4-1 while the application of either alone had no effect ($p = 0.93, 0.99$, respectively; Fig. 2-1G). This indicates that ligand-binding plus a rise in intracellular calcium is necessary and sufficient to induce recruitment of Nedd4-1 to synapses. Taken together, these data indicate that the subcellular distribution of Nedd4-1 is dynamically regulated by specific synaptic cues.

Nedd4-1's C2 domain regulates its trafficking and function in neurons

In addition to the enzymatic HECT domain and four WW domains, Nedd4-1 contains a C2 domain. In a number of other proteins, this domain serves as a calcium-sensor and a regulator of membrane interactions (Nalefski and Falke, 1996). In the yeast homolog Rsp5, the C2 domain is critical for proper membrane localization (Dunn et al., 2004). In MDCK cells, increased calcium causes a shift in localization of mammalian Nedd4-1 to membrane compartments, an effect that requires the C2 domain (Plant et al., 1997; Plant, 2000). Interestingly, this domain has also been found to be auto-inhibitory, as a Nedd4-1 mutant lacking this region displayed dramatically

enhanced ubiquitination activity (Wang et al., 2010). It has been proposed that calcium reduces the inhibitory control by the C2 domain, possibly by promoting membrane association. To test the role of this domain in neurons, we generated an HA-tagged mutant Nedd4-1 with a heavily truncated C2 domain and expressed this mutant variant in hippocampal neurons via Sindbis virus. Under control conditions, Nedd4-1 Δ C2 appears to have a very similar cellular distribution to the wild-type form (Fig. 2-2A). However, we observed no differences in AMPA-treated neurons compared to untreated neurons ($p = 0.72$), indicating that this domain is necessary for AMPA-induced redistribution of Nedd4-1 (Fig. 2-2A, B).

To delineate between its role in synaptic redistribution and an auto-inhibitory role of Nedd4-1's C2 domain, we compared the level of decreased synaptic strength between overexpressed wild-type Nedd4-1 and Nedd4-1 Δ C2 in cultured hippocampal neurons by recording miniature excitatory post-synaptic currents (mEPSCs). As previously reported, overexpression of wild-type Nedd4-1 caused a significant decrease in the amplitude of mEPSCs (Schwarz et al., 2010), compared to control GFP-expressing cells (Fig. 2-2C-E). In contrast, expression of the Nedd4-1 Δ C2 mutant did not cause any reduction of mEPSC amplitude (GFP: 15.8 ± 0.6 pA, $n = 25$ cells; WT: 13.2 ± 0.6 pA, $n = 20$ cells; Δ C2: 16.2 ± 0.7 pA, $n = 23$ cells; $p < 0.05$; Fig. 2-2C-E). There was no observable difference in the frequency of mEPSCs (GFP: 373.2 ± 70.0 ms; WT: 806.5 ± 203.1 ms; Δ C2: 650.1 ± 155.6 ms). To verify that these changes in synaptic strength reflect changes in surface AMPAR expression, we expressed these constructs in hippocampal neurons and immunostained for surface GluA1-containing AMPARs. We observed a reduction in surface GluA1 in Nedd4-1

wild-type expressing cells, but no reduction was observed in Nedd4-1 Δ C2 expressing cells (GFP, normalized surface signal: 1.0 ± 0.05 , $n = 59$ cells; WT: 0.82 ± 0.05 , $n = 57$ cells; Δ C2: 0.95 ± 0.06 , $n = 59$ cells; $p < 0.05$; Fig. 2-2F, G). Therefore, while the Nedd4-1 Δ C2 mutant has been reported to have dramatically enhanced ligase activity (Wang et al., 2010), these data indicate that the C2 domain is required for down-regulating synaptic strength upon overexpression. We further corroborated this by biochemically assessing whether the C2 domain is critical for interaction with GluA1 in co-transfected HEK293 cells. We detected a much stronger interaction of GFP-GluA1 with wild-type HA-Nedd4-1 when compared to the Nedd4-1 Δ C2 mutant, suggesting that the C2 domain is also required for normal binding of Nedd4-1 to AMPA receptors (Fig. 2-2H).

Nedd4-1 is necessary for homeostatic downscaling of synaptic strength

Homeostatic plasticity involves the ability of synapses to dynamically scale their strength up or down in response to prolonged changes in activity levels (Pozo and Goda, 2010; Davis, 2013). Raising activity levels through long-term treatment with the GABA_A receptor antagonist bicuculline leads to a downscaling of synaptic strength due to the removal of synaptic AMPARs (Turrigiano et al., 1998). Several proteins have been shown to be directly or indirectly involved in homeostatic downscaling of AMPARs, with the activity and/or levels of these molecules being regulated by synaptic activity (Ehlers, 2003; Seeburg et al., 2008; Fu et al., 2011). Due to Nedd4-1's role in internalization of AMPARs, we sought to explore whether this E3 ligase plays a role in homeostatic downscaling. We first examined whether a chronic

increase in activity would lead to enhanced AMPAR ubiquitination. Indeed, we observed that neuronal cultures treated with bicuculline for 24h had significantly increased AMPAR ubiquitination (Fig. 2-3A).

We next asked if chronic activity elevation would alter Nedd4-1 protein levels. As expected, total GluA1 levels were significantly reduced in neurons following a 3-day bicuculline treatment (normalized bicuculline level: 0.68 ± 0.07 ; $p < 0.01$; Fig. 2-3B, C). In contrast, bicuculline caused a significant increase in total Nedd4-1 protein levels (1.26 ± 0.09 ; $p < 0.01$; Fig. 2-3B, C). Since we found that the distribution of Nedd4-1 can be modulated by AMPAR activation, we tested whether chronic application of bicuculline induces a change in subcellular distribution of Nedd4-1. We treated HA-Nedd4-1-expressing hippocampal neurons with bicuculline (24h) and observed a significant redistribution of Nedd4-1 to dendritic spines ($p < 0.001$; Fig. 2-3D, E) comparable to that observed with AMPAR activation (Fig. 2-1B). Thus, prolonged bicuculline-induced activity elevation increases AMPAR ubiquitination and up-regulates total and synaptic Nedd4-1 protein levels.

We therefore asked if Nedd4-1 is necessary for homeostatic loss of surface AMPARs and downscaling of synaptic strength. We utilized a Nedd4-1 shRNA hairpin which we previously showed to efficiently knock down endogenous Nedd4-1 protein levels (Schwarz et al., 2010). Hippocampal neurons transduced with control or Nedd4-1 shRNA hairpin lentivirus (co-expressing GFP) for 5 days were subsequently treated with bicuculline or vehicle (DMSO) for 3 days. GFP-positive cells were identified by epi-fluorescence and whole-cell mEPSCs were recorded. As previously reported, chronic treatment with bicuculline caused a significant reduction in both

mEPSC amplitude and frequency in control cells (Ctrl-Veh, amplitude: 16.1 ± 0.5 pA, IEI: 403.1 ± 38.9 ms, $n = 46$ cells; Ctrl-Bic, amplitude: 12.7 ± 0.5 pA, IEI: 876.9 ± 77.7 ms, $n = 46$ cells; $p < 0.001$; Fig. 2-4A-C). Strikingly, we observed that knockdown of Nedd4-1 prevented the bicuculline-induced decrease in mEPSC amplitude (RNAi-Veh, amplitude: 15.9 ± 0.5 pA, $n = 42$ cells; RNAi-Bic, amplitude: 15.3 ± 0.8 pA, $n = 41$ cells; $p = 0.50$; Fig. 2-4A-C). Nedd4-1 knockdown only partially reduced the increase in inter-event interval (RNAi-Veh, IEI: 500.8 ± 68.7 ms; RNAi-Bic: 729.8 ± 86.2 ms; $p < 0.05$). We additionally evaluated whether loss of Nedd4-1 could prevent bicuculline-induced loss of surface AMPARs. While bicuculline caused a significant decrease in surface GluA1 levels in control cells (Ctrl-Veh, normalized surface GluA1 expression: 1.00 ± 0.05 , $n = 57$ cells; Ctrl-Bic: 0.63 ± 0.03 , $n = 60$ cells; $p < 0.001$), the knockdown of Nedd4-1 completely prevented this decrease (RNAi-Veh: 0.88 ± 0.05 , $n = 62$ cells; RNAi-Bic: 0.79 ± 0.05 , $n = 59$ cells; $p = 0.18$; Fig. 2-4E, F). These data indicate Nedd4-1 is required for homeostatic downscaling of synaptic strength.

Nedd4-1 and AMPAR ubiquitination are altered in an Alzheimer's disease model

While the late stages of Alzheimer's disease (AD) are characterized by loss of neurons throughout the brain and severe cognitive impairment, it is theorized that the earliest changes begin at synapses (Selkoe, 2002). Specifically, it has been hypothesized that AMPARs at excitatory synapses are being abnormally internalized, leading to reductions in synaptic strength and eventually total synapse loss (Hsieh et al., 2006). Though there is evidence for this in the literature, the exact mechanism of

this aberrant endocytosis has not been pinned down. Since we have identified a role for Nedd4-1 in promoting AMPAR endocytosis, we theorized that it could be a part of the mechanism responsible for these early synaptic defects. Additionally, a recent paper revealed that levels of Nedd4-1 are increased in brain tissue collected from AD patients, and that treatment of cultured neurons with amyloid beta, the peptide thought to be responsible for many of the disease's effects on the nervous system, increase total levels of Nedd4-1 (Kwak et al., 2012). As such, we sought to examine whether or not treatments of amyloid beta engage Nedd4-1 at the synapse and how AMPARs are affected by this.

We first set out to recapitulate a variety of A β -induced synaptic defects in our cultured neuron system. We utilized two different sources of A β to accomplish this: naturally-secreted A β (both monomeric and oligomeric) in the media collected from Chinese hamster ovary (CHO) cells expressing human APP751 with the Val717Phe mutation (termed 7PA2, with media from non-APP-expressing CHO cells as a control), and synthetic human oligomerized A β_{1-42} with DMSO as a control. A six-day treatment of cultured hippocampal neurons with 7PA2 media resulted in a significant reduction in both mEPSC amplitude and frequency, indicating a weakening and loss of synapses (CHO, amplitude: 16.44 ± 0.77 pA, frequency: 4.66 ± 0.48 Hz, $n = 15$ cells; 7PA2, amplitude: 13.32 ± 0.85 pA; frequency: 1.59 ± 0.34 Hz, $n = 10$ cells; $p = 0.014$ and $p < 0.001$, respectively; Fig. 2-5A-C). To test whether this reduction was specifically due to the presence of oligomeric A β , we utilized the synthetic oligomers and observed that a three-day treatment was sufficient to significantly reduce amplitude, though no effect on frequency was observed (DMSO, amplitude: $22.37 \pm$

2.41 pA, frequency: 2.15 ± 0.73 Hz, $n = 7$ cells; oligomeric A β , amplitude: 13.66 ± 0.79 pA, frequency: 1.57 ± 0.34 Hz, $n = 9$ cells; $p = 0.002$ and $p = 0.723$, respectively; Fig. 2-5D).

Additionally, as has been reported previously, we observed a reduction in dendritic spine density after 3 and 6 days of 7PA2 treatment, which supports the claim that synapses have been lost in this treatment paradigm (CHO 3d: 1.01 ± 0.04 spines/ μm , $n = 39$ dendrites; 7PA2 3d: 0.87 ± 0.04 spines/ μm , $n = 36$ dendrites; $p = 0.011$; CHO 6d: 0.93 ± 0.04 spines/ μm , $n = 68$ dendrites; 7PA2 6d: 0.7 ± 0.03 spines/ μm , $n = 70$ dendrites; $p < 0.001$; Fig. 2-5E-F).

Since previous work has suggested that surface AMPARs become internalized following A β treatment, we next examined the abundance of surface GluA1 in cultured neurons treated with 7PA2 media for three days. Using surface biotinylation, we determined that GluA1 is reduced from the surface of neurons, with total levels unchanged (normalized surface level: 0.81 ± 0.005 , $n = 5$, $p < 0.001$; normalized total level: 0.9 ± 0.04 , $n = 5$, $p > 0.05$; Fig. 2-5G-H). This data supports a model wherein receptors are internalized and synaptic strength is weakened following treatment with A β oligomers.

We next sought to determine the mechanism through which such AMPAR removal may be occurring. As described previously, activation of AMPARs by an agonist or through prolonged activity increase leads to the ubiquitination of AMPARs, causing them to be internalized and prevented from returning to the synaptic surface. To determine whether receptor ubiquitination could be a component of A β -induced synaptic weakening, we examined agonist-induced AMPAR ubiquitination after a pre-

incubation with 7PA2 media. While 7PA2 alone did not induce obvious receptor ubiquitination, it strongly enhanced the ubiquitination triggered by a brief AMPA treatment (Fig. 2-5I). This suggests that A β may be engaging the ubiquitin system at the synapse and making it more responsive to synaptic stimuli.

Since Nedd4-1 has been identified as the main ligase responsible for GluA1 ubiquitination, we next examined whether this ligase was being affected by our treatments. As we observed with chronic activity elevation, a 24-hr treatment with A β caused Nedd4-1 to redistribute within cultured neurons (CHO: 10.4 ± 1.06 local maxima; 7PA2: 18.88 ± 1.31 local maxima; $n \geq 30$ dendrites; $p < 0.001$; Fig. 2-6A-B). A close examination of localization within dendrites revealed that Nedd4-1 is being specifically recruited to dendritic spines after A β treatment (7PA2, fraction of HA signal in spines normalized to CHO: 1.32 ± 0.08 , $n \geq 30$ dendrites; $p = 0.002$; Fig. 2-6C). In agreement with this, we also used surface biotinylation to demonstrate that the amount of surface-associated Nedd4-1 is increased after A β treatment (Fig. 2-6D). Taken together, these data indicate that A β promotes the ubiquitination of AMPARs at the synapse, potentially via relocation of the ubiquitin ligase Nedd4-1

Nedd4-1 is necessary for the synaptic weakening induced by amyloid beta

Since we demonstrated that Nedd4-1 gets recruited to synapses and may potentially be responsible for the ubiquitination and internalization of AMPAR receptors observed in this model, we next asked whether the ligase is an essential component of A β 's ability to weaken synapses. To do this, we utilized a lentiviral Nedd4-1 RNAi construct to reduce Nedd4-1 protein levels in a subset of cultured

neurons. We observed that A β treatments cause a significant reduction in mEPSC amplitude in cells infected with a control (GFP-expressing) virus (GFP+DMSO: 16.75 ± 0.69 pA, $n = 23$ cells; GFP+A β : 13.22 ± 0.35 pA, $n = 23$; $p < 0.001$), but the knockdown of Nedd4-1 prevents the ability of A β treatments to reduce mEPSC amplitude (RNAi+DMSO: 18.03 ± 1.01 pA, $n = 19$; RNAi+A β : 17.78 ± 0.54 pA, $n = 24$; $p = 0.8$; Fig. 2-7A-C). In line with this, our surface biotinylation assay indicated that A β reduces surface GluA1 levels in control neurons (GFP+7PA2 normalized level: 0.85 ± 0.04 , $n = 5$, $p < 0.001$), but not in neurons with reduced Nedd4-1 levels (RNAi+7PA2 normalized level: 1.06 ± 0.06 , $n = 5$, $p = 0.3$; Fig. 2-7D-E).

As mentioned previously, treatments with A β typically result in a reduction of dendritic spine density, indicated a loss of excitatory synapses. Nedd4-1 has been implicated in the control of synaptic strength, but not in the regulation of synapse or spine number. Interestingly, we observed that spine density is reduced in A β -treated control neurons (GFP+CHO: 0.72 ± 0.02 spines/ μm , $n = 52$ dendrites; GFP+7PA2: 0.55 ± 0.02 spines/ μm , $n = 46$ dendrites; $p < 0.001$), but not in Nedd4-1 RNAi-expressing neurons (RNAi+CHO: 0.6 ± 0.02 spines/ μm , $n = 64$ dendrites; RNAi+7PA2: 0.59 ± 0.03 spines/ μm , $n = 53$ dendrites; $p = 0.83$; Fig. 2-7F-G). This could mean that preventing the initial ubiquitin-dependent receptor loss stops the loss of dendritic spines from occurring. Taken together, these results indicate that the ubiquitin ligase Nedd4-1 plays a critical role in the synaptic weakening induced by A β treatments.

2.3: Conclusions

In this collection of experiments, we demonstrate that the ubiquitin ligase Nedd4-1 plays a crucial role in allowing neurons to respond to changes in activity and synaptic environment and adjust the strength of synapses via ubiquitination. Nedd4-1 can be recruited to excitatory synapses following an increase in synaptic activity, putting it into a position to directly regulate surface AMPARs. The homeostatic form of plasticity termed synaptic scaling engages this recruitment mechanism and relies on the presence of Nedd4-1, as loss of this protein prevents activity-induced downscaling. Additionally, this ligase is also redistributed in response to treatment with amyloid beta, the pathogenic compound implicated in the progression of Alzheimer's disease. Loss of Nedd4-1 prevents the reductions in synaptic strength, surface GluA1, and spine density typically caused by A β , which suggests that misregulation of this ligase may in part underlie disease progression.

2.4: Acknowledgments

Chapter 2 contains research published in the following article:

Scudder, S.L., Goo, M.S., Cartier, A.E., Molteni, A., Schwarz, L.A., Wright, R., Patrick, G.N. "Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8." 2014. *The Journal of Neuroscience*: 34(50): 16637-49.

Marisa Goo provided biochemical data and scientific guidance, Anna Cartier and Alice Molteni provided biochemical data (present in the published paper but not in this dissertation), and Rebecca Wright contributed some electrophysiology data (present in

the published paper but not in this dissertation). Part of the research plan originated from Lindsay Schwarz during her time in the lab. Chapter 2 also includes data from the following article:

Rodrigues, E.M., Scudder, S.L., Goo, M.S., Patrick, G.N. “A β -induced synaptic alterations require the E3 ubiquitin ligase Nedd4-1.” 2016. *The Journal of Neuroscience*. In Press.

Elizabeth Rodrigues, a former postdoc in the lab, was the primary author and leader of the research project, and provided all of the biochemistry data. Spine density data was obtained by Marisa Goo.

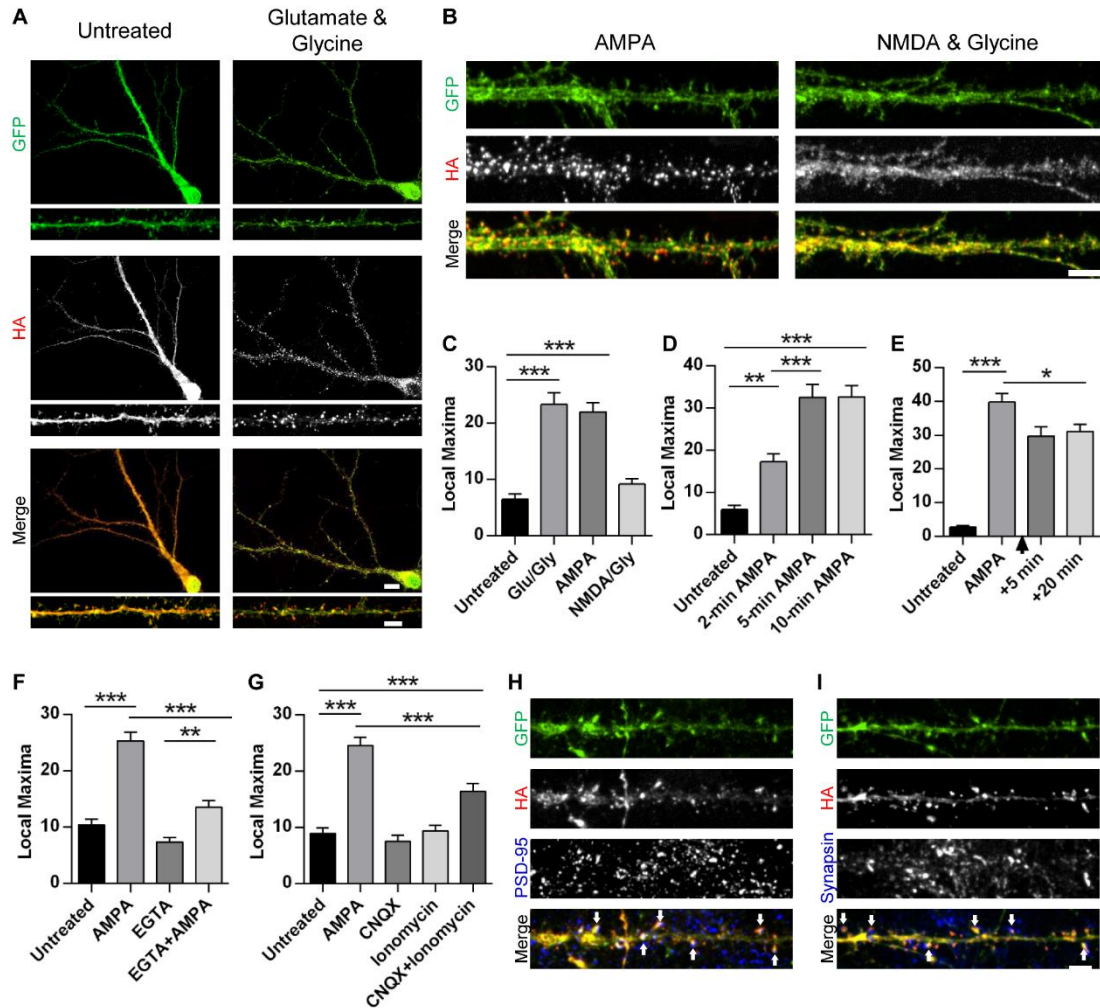


Figure 2-1: AMPAR activation leads to rapid redistribution and accumulation of Nedd4-1 at synapses. **A, B**, Representative immunofluorescent images of dissociated hippocampal neurons 19-22 DIV expressing HA-tagged Nedd4-1 (Sindbis) for 18-20 hours and treated with glutamate and glycine (100 μ M/10 μ M), AMPA (10 μ M), or NMDA and glycine (25 μ M/10 μ M) for 10 minutes, or left untreated and then stained with anti-HA (red) and anti-GFP (green) antibodies. Representative maximum z-projected confocal images of whole cell and dendrite (**A**) or dendrite alone (**B**) are depicted. **C-G**, Quantification of HA-Nedd4-1 fluorescence local maxima: after various drug treatments described in **A** (**C**), time course of AMPA treatments (in minutes) (**D**), persistence of effect 5 or 10 minutes after washout of 10-minute 10 μ M AMPA treatment (indicated by arrowhead) (**E**), effect of calcium chelation with EGTA (2.5 mM, 15-minute pre-treatment) (**F**), and treatments with CNQX (40 μ M, 10 minutes) and ionomycin (10 μ M, 15 minutes) (**G**); $n > 20$ dendrites per condition over 3-4 independent experiments. **H**, Representative immunofluorescent images of dendrites from dissociated hippocampal neurons expressing HA-tagged Nedd4-1 (Sindbis) after AMPA stimulation (10 μ M, 10 minutes) and immunostained with anti-HA (red) and anti-PSD-95 (blue) antibodies. Co-localization of HA and PSD-95 punctate clusters is highlighted by arrow heads. **I**, Representative immunofluorescent images of dendrites after AMPA stimulation (10 μ M, 10 minutes), immunostained with anti-HA (red) and anti-synapsin I (blue) antibodies. Areas of close HA and synapsin proximity are highlighted by arrowheads. $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA with Tukey's *post hoc* analysis. Graphs show mean $\pm$ SEM. Scale bar, 10 μ m and 5 μ m for whole cell and dendrite images, respectively.

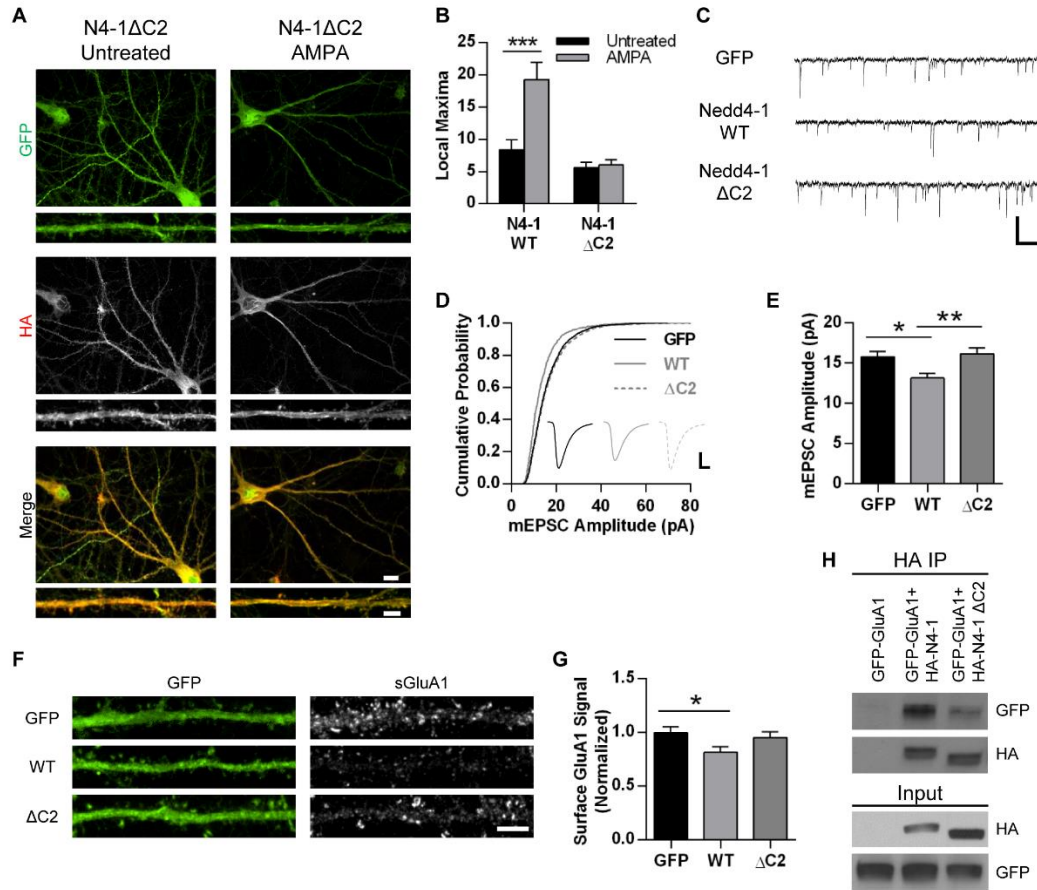


Figure 2-2: Nedd4-1's C2 domain regulates its trafficking and function in neurons. A, Representative immunofluorescent images of dissociated hippocampal neurons 19-22 DIV expressing HA-tagged Nedd4-1ΔC2 (Sindbis) for 18-20 hours and treated with AMPA (10 μM, 10 minutes) or left untreated, and stained for HA (red) and GFP (green). Representative maximum z-projected confocal images of whole cell and dendrite are depicted. Scale bar, 10 μm and 5 μm for whole cell and dendrite images, respectively. **B,** Quantification of images from HA-Nedd4-1 wild-type and ΔC2-expressing cells with and without AMPA (10 μM, 10 minutes); ***p < 0.001, Student's t-test; n > 18 dendrites per condition over 3 independent experiments. **C,** Representative traces of mEPSCs recorded from dissociated hippocampal neurons 18-24 DIV expressing control GFP, HA-Nedd4-1 wild-type, or HA-Nedd4-1ΔC2 (Sindbis) for 18-22 hours. Scale bar, 500 ms and 20 pA. **D,** Cumulative probability distributions of amplitudes of all mEPSCs recorded from neurons infected with Sindbis GFP (control), HA-Nedd4-1 wild-type, or HA-Nedd4-1ΔC2. n = 2739, 1781, and 2426 events, respectively. Inset displays averaged waveform of all events in each condition. Scale bar represents 5 ms, 5 pA. **E,** Mean mEPSC amplitude. *p < 0.05, ANOVA with Tukey's post hoc analysis; n = 20-25 cells; data collected across 3 independent experiments. **F,** Representative immunofluorescent images of hippocampal neurons 18-24 DIV expressing GFP (control), HA-Nedd4-1 wild-type, or HA-Nedd4-1ΔC2 for 18-22 hours and stained for surface GluA1-containing AMPARs (red) and GFP (green). Scale bar, 5 μm. **G,** Quantification of surface GluA1 signal in straightened dendrites of GFP-positive cells in all three conditions. *p < 0.05, ANOVA with Tukey's post hoc analysis; n = 57-59 cells; data collected across 3 independent experiments. **H,** Immunoblots showing reduced co-immunoprecipitation between GFP-tagged GluA1 and HA-tagged Nedd4-1ΔC2 compared to HA-tagged Nedd4-1 WT. HEK293 cells were co-transfected with GFP-GluA1 and either HA-Nedd4-1ΔC2 or HA-Nedd4-1 WT for 18-24 hours. HA-Nedd4-1 was immunoprecipitated with anti-HA antibody and amount of co-precipitating GluA1 was determined through anti-GFP blotting. Graphs show mean ± SEM.

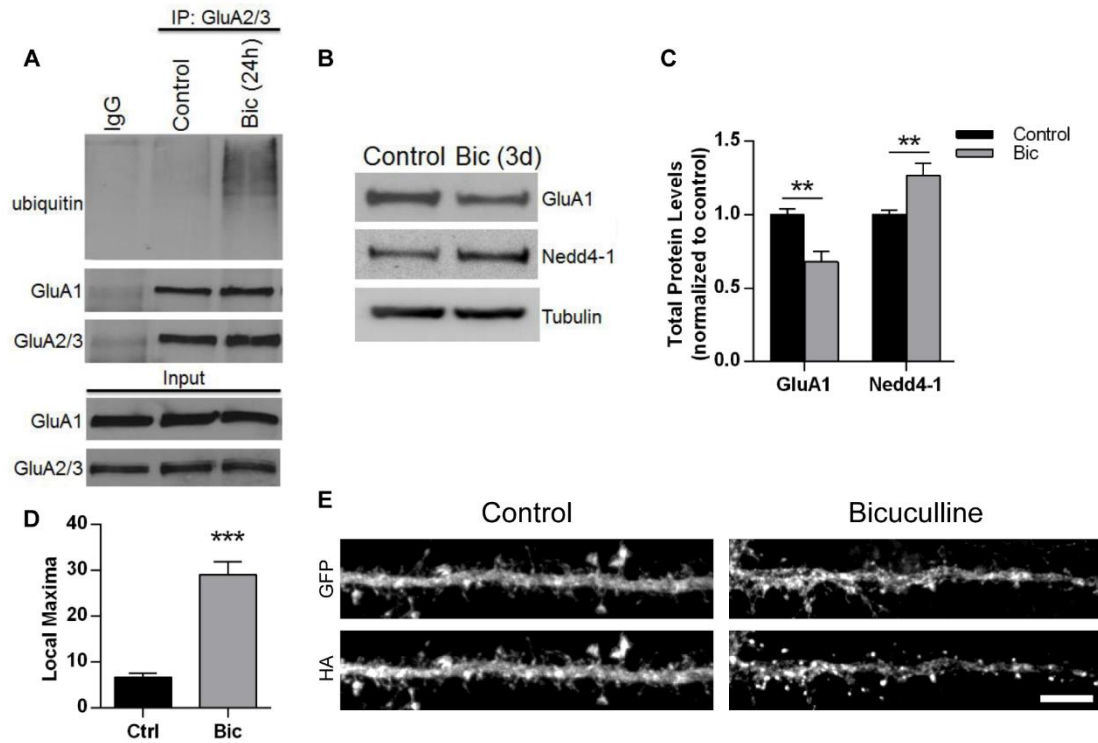


Figure 2-3: Synaptic activity levels regulate Nedd4-1. **A**, Ubiquitination of AMPARs following chronic bicuculline treatment. Cultured cortical neurons 18-22 DIV were treated with bicuculline (20 μ M) for 24 hours before IP with GluA2/3 antibodies in non-denaturing IP conditions. IPs were resolved on SDS-page and probed with anti-ubiquitin, anti-GluA1, and anti-GluA2/3 antibodies. **B**, Chronic bicuculline-induced changes in Nedd4- protein levels. Representative western blots from total lysate of cortical neurons treated with bicuculline (20 μ M) for 72 hours compared to untreated neurons. Cell lysates were resolved on SDS-PAGE and probed anti-GluA1, anti-Nedd4-1, and anti-tubulin antibodies. **C**, Quantification of immunoblots for total GluA1, Nedd4-1, and USP8 levels, normalized to tubulin and control condition, $n > 8$ samples per condition. **D-E**, Chronic application of bicuculline promotes the redistribution of Nedd4-1. Dissociated hippocampal neurons 18-22 DIV expressing GFP HA-Nedd4-1 (Sindbis, 24 hours) were treated with bicuculline (20 μ M, 24 hours) or left untreated and stained with anti-HA (red) and anti-GFP (green) antibodies. Redistribution was quantified using local maxima macro; $n > 20$ dendrites per condition over 2 independent experiments (**D**). Representative maximum z-projected confocal images of whole cell and dendrite are depicted (**E**). Scale bar, 10 μ m and 5 μ m for whole cell and dendrite images, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t-test. Graphs show mean $\pm$ SEM.

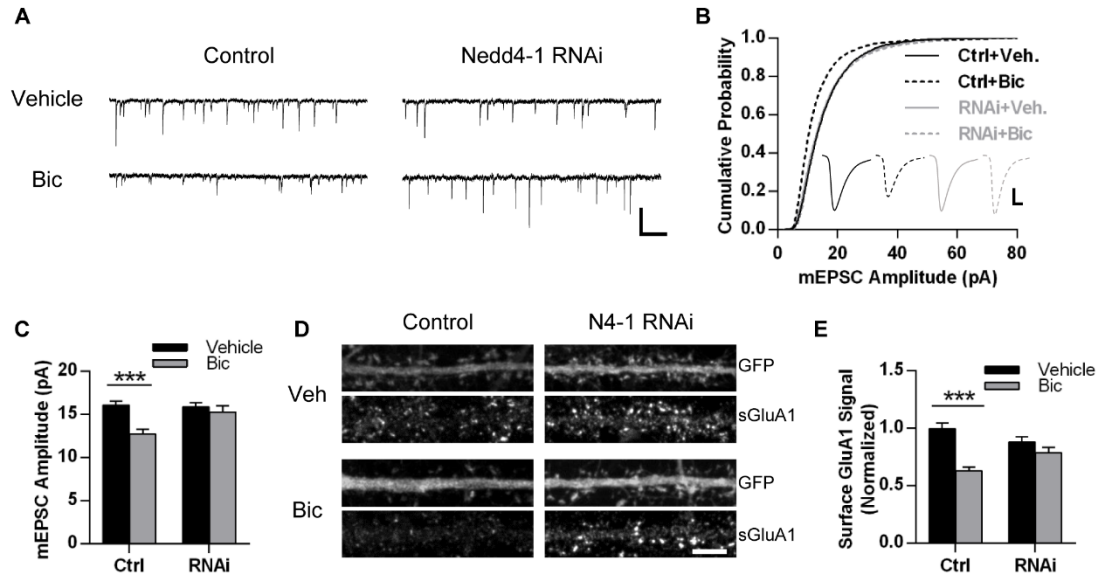


Figure 2-4: Nedd4-1 is required for homeostatic downscaling. **A-C**, Hippocampal neurons DIV 14-15 were infected with FG12 control vector (expressing GFP) or shRNA Nedd4-1 hairpin (co-expressing GFP) for 5 days, and then treated with bicuculline (20 μ M) or vehicle (control, DMSO) for 72 hours. **A**, Representative traces of mEPSCs recorded from each condition as indicated (scale bar represents 500 ms, 20 pA). **B**, Mean mEPSC amplitude for all conditions; $n = 41$ -46 cells across 4 independent experiments. **C**, Cumulative probability distributions of amplitudes of all events recorded from neurons in each condition. $n = 3532, 3180, 3205, 3140$ events. Inset displays averaged waveform of all events in each condition (scale bar represents 5 ms, 5 pA). **D**, Representative immunofluorescence images of straightened dendrites from hippocampal neurons from each condition described in **A**, stained for surface GluA1 (red) and GFP (green). **E**, Quantification of images from dendrites in all conditions, normalized to Ctrl-Veh; $n = 57$ -60 cells across 3 independent experiments. * $p < 0.05$, *** $p < 0.001$; Student's t-test. Graphs show mean $\pm$ SEM.

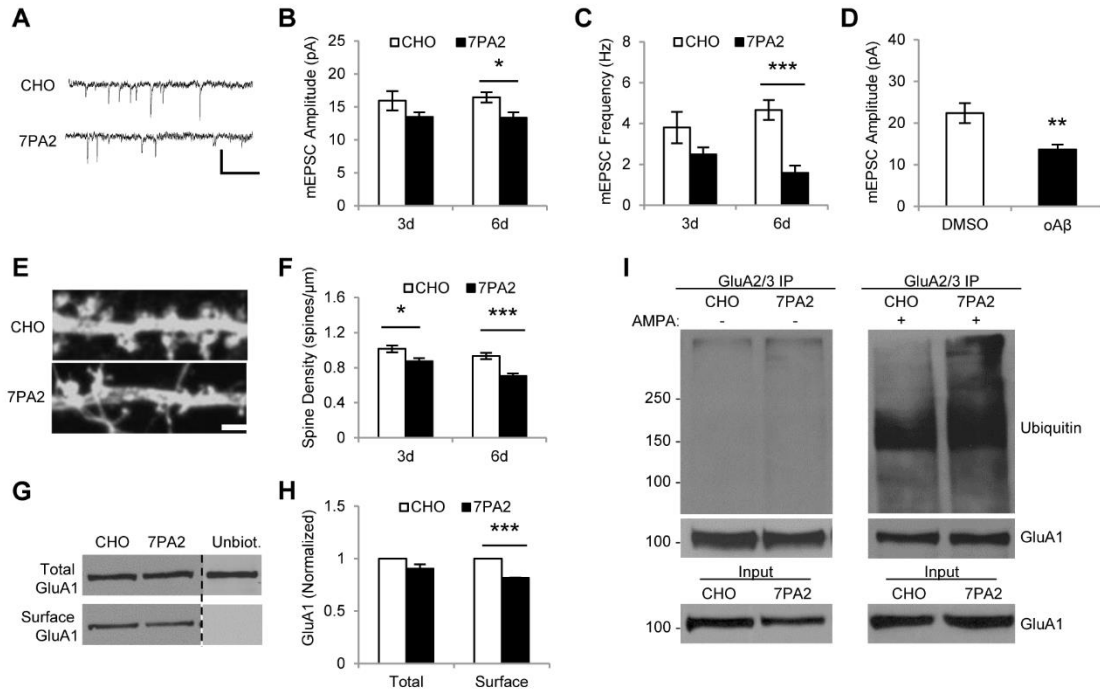


Figure 2-5: Aβ-induced synaptic alterations coincide with enhanced AMPAR ubiquitination. **A**, Representative traces of mEPSCs from each condition indicated. Scale bar = 500 ms, 20 pA. **B**, **C**, Mean mEPSC amplitude (**B**) and frequency (**C**) for neurons (DIV 16-20) treated with CHO or 7PA2 CM for 3 or 6 days. **D**, Mean mEPSC amplitude for neurons treated with DMSO or oligomeric Aβ for 3 days. **E**, Representative immunofluorescent images of dendrites treated with CHO or 7PA2 CM for 6 days. Scale bar = 5 μm. **F**, Quantification of spine density from conditions displayed in **E**. **G**, Representative Western blot of total and biotinylated surface GluA1 levels in neurons treated with CHO or 7PA2 CM for 3 days. **H**, Quantification of immunoblots for total and surface GluA1, normalized to CHO. **I**, Representative immunoblot depicting enhanced AMPA-induced (100 μM, 10 min) ubiquitination of AMPARs following a 14-hour treatment with 7PA2 CM. *p < 0.05, **p < 0.01, ***p < 0.001.

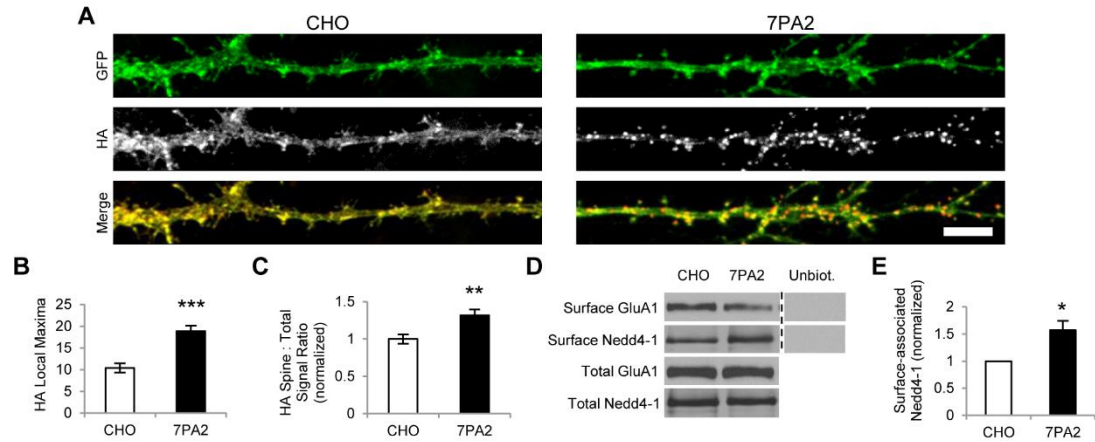


Figure 2-6: A β promotes Nedd4-1 redistribution into dendritic spines and increases Nedd4-1 association with surface proteins. **A**, Representative immunofluorescent images of hippocampal dendrites expressing HA-tagged Nedd4-1, treated with CHO or 7PA2 CM for 23 hours and stained with anti-GFP (green) and anti-HA (white) antibodies. Scale bar = 5 μ m. **B**, Quantification of HA-Nedd4-1 fluorescence local maxima from conditions displayed in **A**. **C**, Normalized ratio of spine to total HA levels between conditions in **A**. **D**, Representative Western blot of surface and total Nedd4-1 and GluA1 in neurons treated with CHO or 7PA2 CM for 3 days. **E**, Quantification of immunoblots for surface-associated Nedd4-1, normalized to CHO. * $p < 0.05$, *** $p < 0.001$.

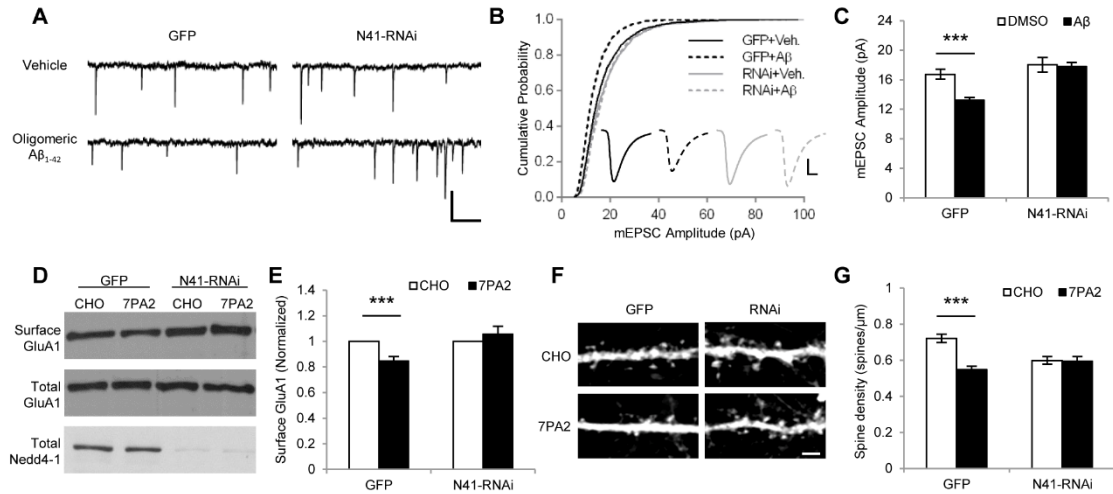


Figure 2-7: Nedd4-1 is necessary for A β -induced synaptic alterations. **A**, Representative traces of mEPSCs from each condition indicated. Scale bar = 500 ms, 20 pA. **B**, Cumulative probability distributions of amplitudes of all mEPSCs recorded from each condition. Inset displays averaged trace for all mEPSCs in each group; n = 1960, 2287, 1468, 2302 events. Scale bar = 5 ms, 5 pA. **C**, Mean mEPSC amplitude for cells in all conditions. **D**, Representative Western blot of surface GluA1 levels in neurons treated with CHO or 7PA2 CM. **E**, Quantification of immunoblots for surface GluA1, normalized to CHO within each virus. **F**, Representative immunofluorescent images of dendritic spines from hippocampal neurons under each of the conditions indicated. Scale bar = 5 μ m. **G**, Quantification of spine density from conditions displayed in **F**. ***p < 0.001.

Chapter 3:

**Synaptic structure and function are
altered by the neddylation inhibitor
MLN4924**

3.1: Introduction

The posttranslational modification of proteins by the ubiquitin-like small molecule NEDD8 has previously been shown to be vital in a number of cell signaling pathways. In particular, conjugation of NEDD8 (neddylation) serves to regulate protein ubiquitination through modifications to a number of E3 ubiquitin ligases, often serving to control their ligase activity. Despite the prevalence of NEDD8 in neurons, very little work has been done to characterize the role of this modifier in these cells. Here, we use the recently developed NEDD8 Activating Enzyme (NAE) inhibitor MLN4924 and report evidence of a role for NEDD8 in regulating mammalian excitatory synapses. Application of this drug to dissociated rat hippocampal neurons caused reductions in synaptic strength, surface glutamate receptor levels, dendritic spine width, and spine density, suggesting that neddylation is involved in the maintenance of synapses.

3.2: Results

Inhibition of the NEDD8 Activating Enzyme reduces synaptic strength

Neddylation is known to be a regulator of a number of functions in diverse cell types, though little work has been done to explore its role in neurons. Using the NEDD8 Activating Enzyme (NAE) inhibitor MLN4924, we investigated the role of this posttranslational modification in the maintenance of neuronal synapses. First, we sought to verify that this drug effectively blocks neddylation in cultured neurons. Dissociated cortical neurons were treated with 1 μ M MLN4924 or vehicle (DMSO) for 6 or 12 hours. Cells were lysed in the presence of the zinc chelator and COPS5

inhibitor 1,10-orthophenanthroline (OPT), which has been shown to allow for detection of neddylated proteins (Bennett et al., 2010). In the control condition, probing the neuronal lysates for NEDD8 yields a number of dark bands which are indicative of neddylated proteins of various molecular weights. Treatment with MLN4924, however, abolishes such bands, demonstrating that this drug prevents the conjugation of NEDD8 to substrates within neurons (Fig. 3-1A).

We next determined whether inhibition of neddylation would affect synaptic strength. Dissociated hippocampal rat neurons were first infected with 2-gene GFP for visualization of cell morphology (16-20 hours total), then treated with 1 μ M MLN4924 or vehicle (DMSO) for 6 hours. Miniature excitatory postsynaptic currents (mEPSCs) were recorded from GFP-positive pyramidal-like cells to determine the effect on synaptic strength. We observed that six hours of MLN4924 treatment caused a significant decrease in the amplitude of mEPSCs (DMSO, amplitude: 15.67 ± 0.88 pA, $n = 17$ cells; MLN4924: 12.60 ± 0.52 pA, $n = 19$ cells; $p < 0.01$; Fig. 3-1B-E). We observed no changes to inter-event interval (DMSO, IEI: 450.9 ± 49.24 ms; MLN4924: 521.4 ± 58.19 ms; $p = 0.37$; Fig. 3-1D), suggesting a postsynaptic alteration. However, since the entire mEPSC amplitude distribution does not fully shift to the left (Fig 3-1E), it is also possible that we are observing preferential selection of weaker synapses over stronger ones, rather than a reduction in the strength of all synapses.

To determine whether a longer treatment with MLN4924 would enhance this reduction, we increased the treatment time to 12 hours. We observed a decrease in mEPSC amplitude comparable to that of the shorter treatment (DMSO, amplitude:

15.05 $\pm$ 0.71 pA, n = 46 cells; MLN4924: 12.83 $\pm$ 0.59 pA, n = 43; p < 0.05; Fig. 3-1B-F) and no change in inter-event interval (DMSO, IEI: 309 $\pm$ 38.35 ms; MLN4924: 371.3 $\pm$ 50.99 ms; p = 0.33; Fig. 3-1D), demonstrating that no further reduction in synaptic strength occurs with longer inhibition of neddylation.

Application of MLN4924 reduces surface GluA1 expression

Changes in mEPSC amplitude are often accompanied by changes in the levels of surface glutamate receptors, particularly AMPA receptors (AMPA receptors). To assess whether these observed changes in mEPSC amplitude are the result of altered postsynaptic surface AMPAR expression, we infected hippocampal neurons with GFP to visualize morphology, treated with MLN4924 or vehicle for 6 hours, and stained for surface GluA1 (an AMPAR subunit) using immunofluorescent antibodies. We observed reduced surface GluA1 fluorescence in MLN4924-treated neurons (DMSO, normalized surface signal: 1.00 $\pm$ 0.04, n = 40 cells; MLN4924: 0.88 $\pm$ 0.03, n = 40 cells; p < 0.05; Fig. 3-2A, B).

To determine whether total GluA1 protein levels are altered after inhibition of neddylation, dissociated cortical neurons were treated with 1 μ M MLN4924 or DMSO for 6 hours and lysed. Lysates were resolved on SDS-PAGE and probed with GluA1 antibodies. We did not observe a significant change in total GluA1 levels after MLN4924 treatment (MLN4924, normalized level: 1.26, n = 3 independent experiments; p = 0.58; Fig. 3-2C). This may indicate that receptors have been removed from synapses but not yet degraded, or that synapses have been dismantled but the component proteins are still present.

Our observations of reduced surface GluA1 and mEPSC amplitude do not appear to be the result of poor neuronal health, as neurons in all conditions appeared healthy and with comparable morphologies (Fig. 3-2D). Taken together, these data indicate that the inhibition of neddylation in hippocampal neurons leads to functional changes that reduce synaptic strength.

Inhibition of neddylation leads to alterations to dendritic spines

The majority of excitatory synapses are located on dendritic spines. In hippocampal pyramidal neurons, there is a strong correlation between the numbers of dendritic spines and excitatory synapses (Nimchinsky et al., 2004). Additionally, spine morphology is closely related to synaptic function (Hering and Sheng, 2001; Matsuzaki et al., 2001). To determine whether the observed functional changes caused by neddylation inhibition are accompanied by structural changes in neurons, we examined the size and density of dendritic spines in dissociated hippocampal neurons treated with 1 μ M MLN4924 for 6 hours. MLN4924 treatment produced a significant decrease in spine width (DMSO, normalized width: 1.00 ± 0.02 , $n = 64$ dendrites; MLN4924: 0.84 ± 0.01 , $n = 64$ dendrites; $p < 0.001$; Fig. 3-3A, D) and density (DMSO, normalized density: 1.00 ± 0.03 ; MLN4924: 0.85 ± 0.02 ; $p < 0.001$; Fig. 3-3C), but did not affect spine length ($p = 0.93$; Fig. 3-3B). We extended our treatment time to 12 hours and observed a similar reduction in width (DMSO, normalized width: 1.00 ± 0.01 , $n = 70$ dendrites; MLN4924: 0.88 ± 0.01 , $n = 79$; $p < 0.001$; Fig. 3-3A, E) and no reduction in density ($p = 0.52$; Fig. 3-3C) or length ($p = 0.67$; Fig. 3-3B), demonstrating that spines do not continue shrinking after the initial reduction in width.

These data indicate that the functional reduction in synaptic strength caused by inhibition of neddylation is accompanied by structural changes to dendritic spines.

3.3: Conclusions

This study reveals a role for the ubiquitin-like protein NEDD8 in regulating synaptic strength. By utilizing a novel inhibitor of NEDD8 conjugation, we demonstrate that loss of neddylation results in reductions in mEPSC amplitude, loss of surface GluA1, and thinning of dendritic spines. While the exact targets of neddylation at the synapse remain poorly defined, our work suggests that this regulatory mechanism is essential in maintaining excitatory synapses.

3.4: Acknowledgments

Chapter 3 contains data from the following article:

Scudder, S.L., Patrick, G.N. “Synaptic structure and function are altered by the neddylation inhibitor MLN4924.” 2015. *Molecular & Cellular Neuroscience*: 65: 52-57.

The dissertation author is solely responsible for the data in this chapter. Dr. Eric Bennett provided helpful reagents and advice for this project.

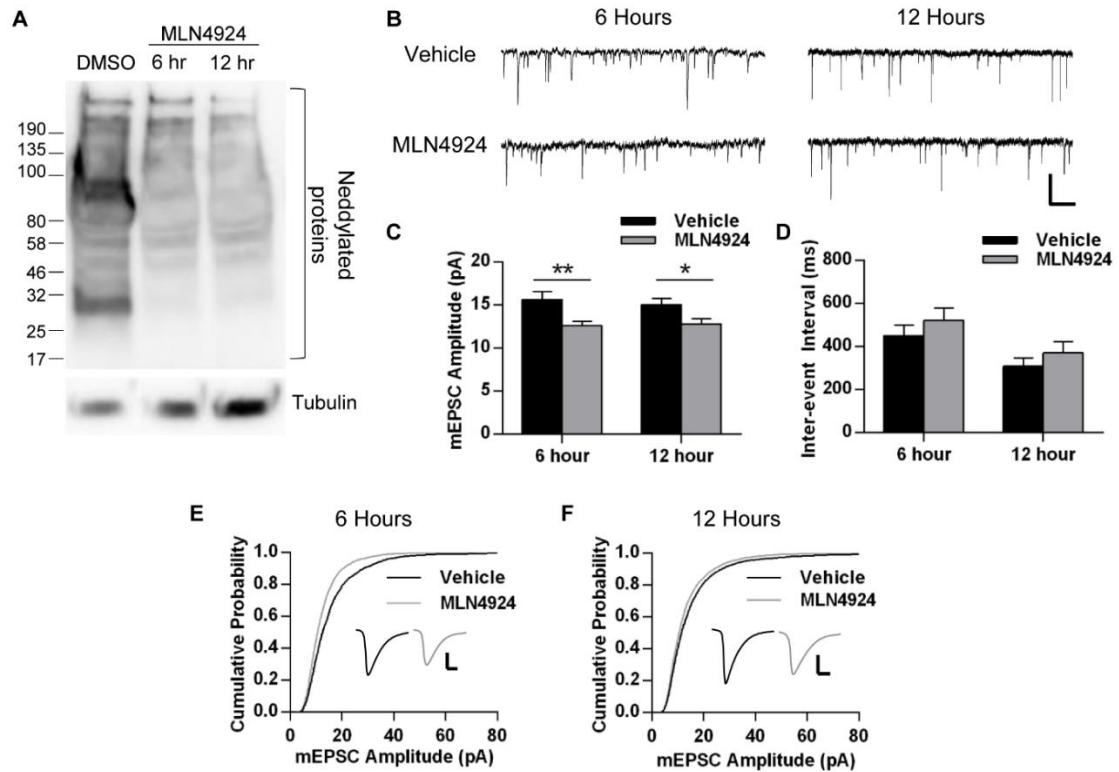


Figure 3-1: Inhibition of neddylation with MLN4924 reduces mEPSC amplitude. **A**, Western blot depicting inhibition of NEDD8 conjugation in neurons after treatment with MLN4924. Dissociated cortical rat neurons (DIV 19-25) were treated with DMSO (vehicle) for 12 hours or with 1 μ M MLN4924 for 6 or 12 hours. Cells were lysed in the presence of 1,10-orthophenanthroline, then lysates were resolved on 4-20% SDS-PAGE and probed for NEDD8 and tubulin ($n = 2$ independent experiments). **B-F**, Dissociated hippocampal rat neurons (DIV 19-25) were infected with 2-gene GFP for 16-20 hours. Neurons were treated with 1 μ M MLN4924 or DMSO (vehicle) for the last 6 or 12 hours of infection and mEPSCs were recorded from GFP-positive pyramidal-like neurons. **B**, Representative mEPSC traces recorded from neurons in each condition. Scale bar represents 500 ms, 20 pA. **C**, Average mEPSC amplitudes of neurons recorded from the four conditions; $n > 19$ cells per condition across 3 independent experiments. **D**, Average inter-event intervals of neurons in each condition. **E**, Cumulative probability plot of all mEPSC events recorded from cells treated with MLN4924 for 6 hours; $p < 0.001$, Kolmogorov-Smirnov test; $n = 2003$, 2224 events. **F**, Cumulative probability plot from mEPSCs in 12-hour conditions; $p < 0.001$, K-S test; $n = 5781$, 5350 events. Insets depict averaged waveform from all events in each condition, scale bar represents 5 ms, 5 pA.. Graphs depict mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, Student's t test.

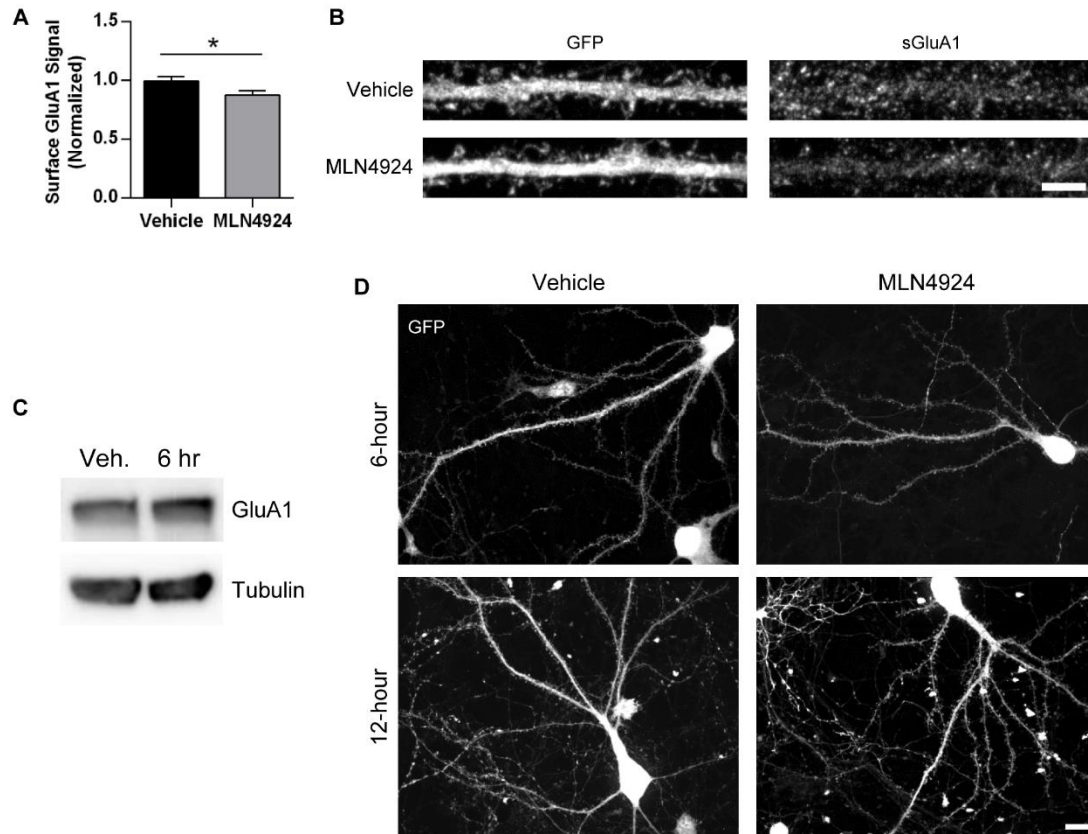


Figure 3-2: MLN4924 reduces surface AMPAR expression. **A**, Cultured hippocampal neurons (DIV 19-25) were infected with 2-gene GFP for 16-20 hours and treated with 1 μ M MLN4924 or DMSO (vehicle) for the last 6 hours of infection. Cells were then fixed and stained for surface GluA1 using immunofluorescent antibodies. Images of GFP-positive neurons were acquired with a confocal microscope and straightened dendrites were analyzed for GluA1 expression. Values were normalized to the vehicle-treated condition; $n = 40$ neurons per condition across two independent experiments; $p < 0.05$, Student's t test. Graph depicts mean $\pm$ SEM. **B**, Representative z-stacked immunofluorescent images of dendrites from vehicle- and MLN4924-treated neurons, stained for GFP and GluA1. Scale bar represents 5 μ m. **C**, Representative Western blot depicting total GluA1 after MLN4924 treatment. Dissociated rat cortical neurons (DIV19-25) were treated with 1 μ M MLN4924 or vehicle for 6 hours, then lysed. Lysates were resolved on 10% SDS-PAGE and probed with GluA1 and tubulin antibodies. **D**, Representative z-stacked images of dissociated hippocampal neurons expressing GFP after 6 and 12 hours of vehicle or MLN4924 treatment. Scale bar represents 10 μ m.

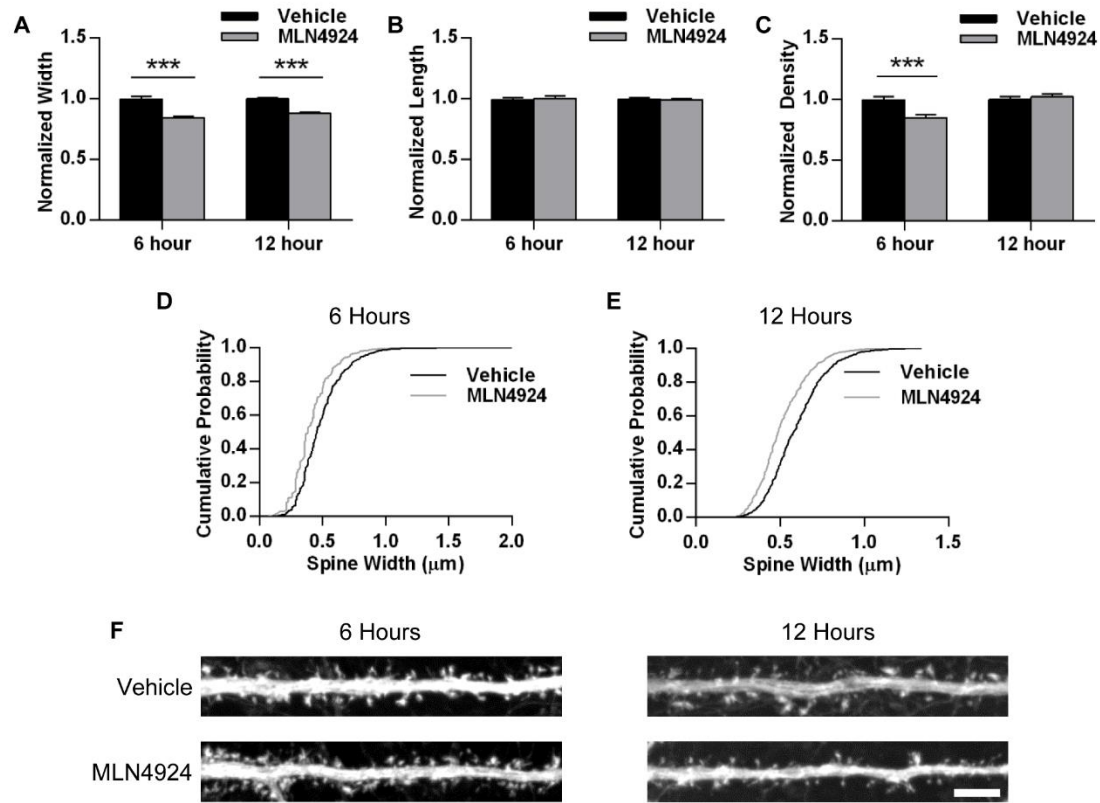


Figure 3-3: Inhibition of neddylation affects dendritic spines. Cultured hippocampal neurons (DIV 19-25) were infected with 2-gene GFP for 16-20 hours and treated with 1 μM MLN4924 or DMSO (vehicle) for the last 6 or 12 hours. Cells were fixed and stained for GFP. GFP-positive cells with pyramidal-like morphology were chosen for analysis. **A**, Average dendritic spine width (across widest point) for dendrites from vehicle- and MLN4924-treated neurons, normalized to vehicle condition. **B**, Average dendritic spine length (from shaft to tip) for dendrites from each condition, normalized to vehicle. **C**, Average spine density for dendrites in each condition, normalized to vehicle. **D**, Cumulative probability plot of width measurements from all spines analyzed from neurons treated with vehicle or MLN4924 for 6 hours; $p < 0.001$, K-S test; $n = 2630, 2244$ spines. **E**, Cumulative probability plot for all spine widths from 12-hour conditions; $p < 0.001$, K-S test; $n = 1274, 1257$ spines. **F**, Representative images of dendrites straightened from GFP-expressing neurons treated with 6 or 12 hours of vehicle or MLN4924. Scale bar represents 5 μm . *** $p < 0.001$, Student's t-test; $n > 60$ dendrites per condition across two independent experiments. Graphs depict mean $\pm$ SEM.

Chapter 4:

**Role of Rpt6 S120 phosphorylation in
synaptic function and plasticity**

4.1: Introduction

As previously described, the 26S proteasome is known to degrade a number of synaptic proteins in a plasticity-dependent manner. Inhibition of the proteasome affects synaptic plasticity, particularly LTP, suggesting that this activity-dependent degradation plays a crucial role in regulating synapses. However, the exact mechanism that allows for such fine control is not yet known.

Multiple studies have shown that proteasomes can undergo activity-induced recruitment to dendritic spines (Bingol & Schuman, 2006; Shen et al., 2007). These complexes can become sequestered there to degrade synaptic proteins, and the kinase CaMKII α appears to be critical in this process. The regulatory Rpt6 subunit of proteasomes contains a serine (S120) known to be phosphorylated by this kinase, and the phosphorylation state of that site varies with synaptic activity and during learning paradigms (Djakovic et al., 2009; Djakovic et al., 2012; Jarome et al., 2013). Interestingly, it has been proposed that the kinase activity of CaMKII α is dispensable in its regulation of proteasome localization, and thus this site would not be of particular importance for plasticity that relies merely on recruitment of proteasomes (Bingol et al., 2010). However, it is not yet known whether that site could regulate other aspects of proteasome function in neurons, such as protease activity, and whether that type of regulation could affect synaptic function and plasticity.

In order to study the role of this phosphorylation site (S120) on proteasomes, synaptic plasticity, and learning and memory, we developed a knock-in mouse with endogenous Rpt6 mutated to a phospho-dead (serine to alanine, S120A) or phospho-mimetic (serine to aspartic acid, S120D) state. These mice allow for a more careful

study of the exact role of proteasome phosphorylation (specifically at serine 120) at the synapse, and also enable us to observe the effects of perturbation in an intact organism. Here, we demonstrate that overall behavior and brain function is not evidently altered with these mutations, and that this phosphorylation site is dispensable in hippocampal LTP and fear conditioning. This suggests that the established role for proteasomes in regulating plasticity and learning must be mediated by an alternate mechanism, or that compensatory mechanisms are recruited upon introduction of these mutations.

4.2: Results

Synaptic transmission is intact in the hippocampi of S120A and S120D animals

While previous studies were conducted in dissociated neurons following overexpression of mutated Rpt6, the generation of S120 knock-in mice enabled us to examine synaptic transmission in a functioning circuit where all neurons are expressing mutated endogenous Rpt6. To determine whether Rpt6 S120 mutations affect basal synaptic transmission in the hippocampus, we generated acute hippocampal slices from young mutant and wild-type mice and conducted field excitatory postsynaptic potential (fEPSP) recordings.

We first assessed whether mutant mice have a typical response curve following increasing intensities of Schaffer collateral (CA3-CA1) stimulation. We observed that slices from both S120D and S120A animals display a normal input-output relationship when compared to slices obtained from age-matched wild-type mice (Fig. 4-1A,E). This indicates that the hippocampal circuit has developed normally and has roughly

the same amount of synaptic connectivity as a wild-type hippocampus. Thus, mutations to S120 do not obviously impact the formation and maintenance of excitatory synapses in the hippocampus, and do not appear to strongly impact synaptic strength at these synapses. To assess whether presynaptic release mechanisms were intact in S120D and S120A mice, we next utilized a paired pulse facilitation (PPF) assay. By stimulating Schaffer collateral axons twice at varying pulse separations, we revealed strong facilitation of fEPSPs in wild-type slices at small separations (50 ms, 100 ms). S120D and S120A slices displayed identical levels of facilitation, indicating that presynaptic neurotransmitter release is intact and has normal responses to increased presynaptic calcium (Fig. 4-1B,F).

Our observation of normal basal transmission conflicted with our previous work that showed alterations in synaptic strength in cultured neurons expressing exogenous S120A and S120D constructs (Djakovic et al., 2012). This could be due to circuit-level compensation in our mutant animals, or the previous results could have been due to complications of overexpressing the Rpt6 subunit. To further investigate this, we generated cultured dissociated hippocampal neurons from P0/1 wild-type and S120D mouse pups and recorded miniature excitatory postsynaptic currents (mEPSCs) from mature pyramidal-like neurons. We observed that mEPSC frequency and amplitude are unaltered in these neurons, in contrast to the previous finding that Rpt6-S120D-expressing neurons exhibit reduced amplitude (Fig. 4-1I-L). Thus, either cellular processes have corrected any deficit caused by our endogenous mutation, or the S120 site does not play a significant role in regulating synaptic strength.

Long-term potentiation is normal in S120A and S120D animals

Since previous work with proteasome inhibitors pointed to an important role for these complexes in mediating LTP, we next sought to determine whether our Rpt6 mutations would affect this plasticity paradigm. We generated acute hippocampal slices from young (P21-P27) age-matched mice and induced LTP in the CA3-CA1 pathway using four trains of 1-second 100 Hz stimulation. We observed that hippocampal slices from S120D and S120A animals undergo normal levels of fEPSP potentiation compared to wild-type mice (Fig. 4-1C,G).

Due to previous studies suggesting that proteasome activity can modulate LTP induction thresholds, we next used a weaker stimulation paradigm, instead stimulating with a single 1-second 100 Hz train. While both mutants displayed normal levels of slight potentiation 15-30 minutes after LTP induction (Fig. 4-1D,H), we did observe a suppression of post-tetanic potentiation and early LTP in S120D animals (Fig. 4-1D). However, since potentiation eventually reached wild-type levels, it appears that neither mutation has a strong effect on LTP.

Rpt6 mutants undergo normal cue- and context-induced fear conditioning

To further examine the role of Rpt6 phosphorylation in neurons, we next tested the mice on learning paradigms. We utilized Pavlovian fear conditioning to assess learning in our mutants, which can be used to test both immediate and long-term memory. In this paradigm, mice are administered a shock that is paired with an auditory cue, and this cue or the context of the shock (the chamber) can later be used to evoke a fear response, measured as freezing. Both cued and contextual fear learning

rely on intact plasticity in the amygdala, while contextual fear learning additionally requires the hippocampus. Previous studies indicated that proteasome-dependent degradation plays a crucial role in fear learning (Jarome & Helmstetter, 2013). However, the lack of an LTP deficit in our mutants suggested that synaptic plasticity is intact despite our mutations, making it important to directly test fear conditioning in the mutant mice.

The S120D mutants displayed normal baseline motor behavior ($F(1,32) = 1.25$, $p = 0.27$; $n = 13$ (WT), 21 (S120D); Fig. 4-2A), though the shock reactivity of S120D mice was slightly reduced compared to wildtype mice ($F(1,32) = 10.48$, $p < 0.005$; Fig. 4-2A). Similarly, S120A mice exhibited normal baseline activity ($F(1,22) = 0.25$, $p = 0.62$; $n = 8$ (WT), 16 (S120A); Fig. 4-2E) while also displaying slightly reduced shock reactivity ($F(1,22) = 4.33$, $p = 0.049$; Fig. 4-2E). Despite the reduced shock reactivity, we observed intact immediate memory in both mutants (S120D vs WT: $F(1,32) = 0.004$, $p = 0.95$; S120A vs WT: $F(1,22) = 1.29$, $p = 0.27$; Fig 4-2B,F). When contextual fear memory was assessed 24 hours later, normal levels of freezing were observed in both phospho-mimetic and phospho-dead mutants (S120D vs WT: $F(1,32) = 0.087$, $p = 0.77$; S120A vs WT: $F(1,22) = 1.645$, $p = 0.21$; Fig. 4-2C,G). Cue-induced freezing at this time-point was also intact in all genotypes (S120D vs WT: $F(1,32) = 1.20$, $p = 0.28$; S120A vs WT: $F(1,22) = 0.119$, $p = 0.73$; Fig. 4-2D,H), indicating that fear conditioning is not affected by mutations to Rpt6 S120.

S120 phospho-status does not affect Rpt6 localization in neurons

The exact relationship between S120 phosphorylation and proteasome localization remains unknown, as conflicting data has been reported. CaMKII α kinase activity has been reported to be dispensable for proteasome trafficking (Bingol et al., 2010), but it has also been reported that the phospho-status of this site can regulate co-localization with PSD95 and may affect tethering to the actin cytoskeleton (Djakovic et al., 2012). The S120 mutant mice afforded us the ability to explore the relationship between S120 phospho-status and Rpt6 localization in a direct way. Using cultured hippocampal neurons generated from phospho-mimetic (S120D) and wild type mice, we explored proteasome distribution in dendrites. Use of a Rpt6 antibody revealed that this subunit is similarly localized in S120D and wild type neurons, with a proportion of the signal being localized within dendritic spines (Fig. 4-3A,C). Additionally, there was no change to co-localization of Rpt6 with the postsynaptic protein PSD95 (Fig. 4-3B). We also observed that PSD95 intensity within spines remained the same in S120D neurons, further supporting the previous observations that excitatory synapses are intact (Fig. 4-3D). Thus, the cellular distribution of proteasomes within neurons may be governed by mechanisms that do not involve CaMKII α -dependent phosphorylation of Rpt6, though this does not rule out the possibility of this site being critical in activity-dependent trafficking of proteasomes.

4.3: Conclusions

The wealth of evidence supporting a role for dynamic proteasome-dependent protein degradation in plasticity and memory led us to generate the first mouse model

with an alteration to proteasome function. Mutating serine 120 of the proteasome subunit Rpt6 to an alanine (S120A, phospho-dead) or aspartic acid (S120D, phospho-mimetic) had no effect on mouse viability or brain development. Basal synaptic transmission appeared normal in both mutant genotypes, and long-term potentiation is also fully intact. In line with this, our mutant mice displayed no memory impairments on cued or contextual fear conditioning. This data suggests that CaMKII-dependent Rpt6 phosphorylation does not play a critical role in regulating synaptic strength, LTP, or fear learning.

4.4: Acknowledgments

Chapter 4 is currently being prepared for submission and the final paper will include substantial data from Frankie Gonzales, who will serve as co-first-author on the manuscript. Kristin Howell (a graduate student in Stephan Anagnostaras's lab) collected all behavioral data from the mutant mice and the generation and basic characterization of the mice was conducted by Lara Dozier. The tentative citation is as follows:

*Gonzales, F.R., *Scudder, S.L., Howell, K.H., Anagnostaras, S.G., Patrick, G.N.

“Rpt6 S120 mutations do not affect synaptic plasticity and learning.”

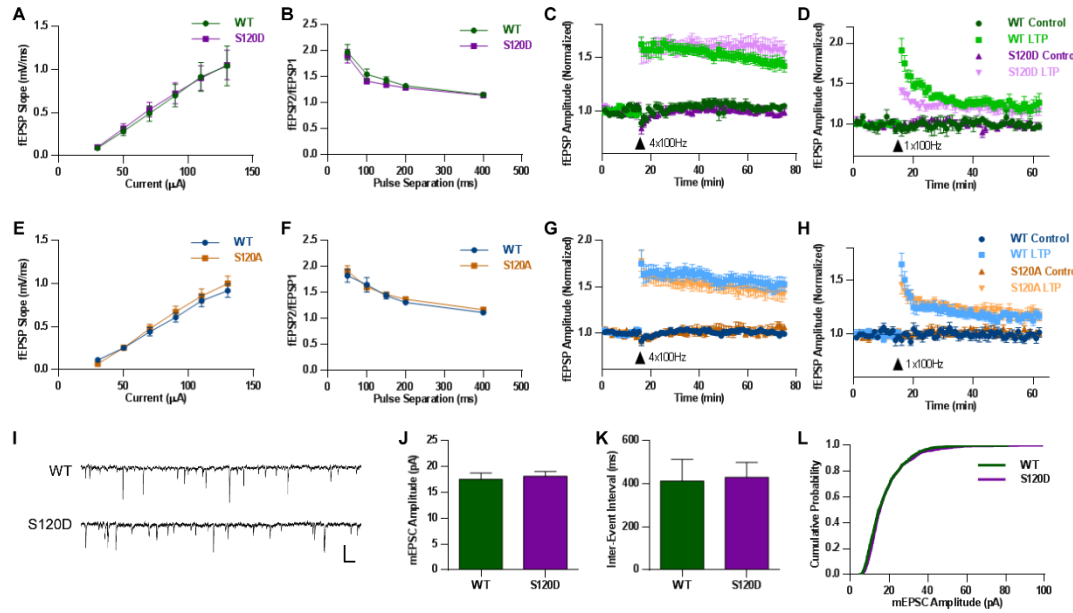


Figure 4-1: Hippocampal synaptic transmission and long-term potentiation are unaltered in S120 knock-in mice. **A**, Input/output curve depicting increased field EPSP responses to increased current amplitude in acute hippocampal slices from wildtype and S120D mice ($n = 11$ slices per genotype). **B**, Two successive stimuli with short separation delivered to the Schaffer collateral of acute hippocampal slices leads to enhancement of fEPSP amplitude (paired pulse facilitation) in wildtype and S120D mice ($n = 10$ slices). **C**, Delivery of 4 1-second trains (20 s apart) of 100 Hz stimulation causes potentiation of fEPSP amplitude in acute hippocampal slices from wildtype and S120D mice ($n = 9, 10$ slices). **D**, Delivery of a single 1-second 100 Hz train induces short-lasting post-tetanic potentiation followed by a small long-lasting potentiation of fEPSP amplitude ($n = 5, 6$ slices). **E**, Input/output curve in slices from wildtype and S120A mice ($n = 15$ slices per genotype). **F**, Paired pulse facilitation in wildtype and S120A slices ($n = 16$ slices). **G**, LTP induction in response to 4 1-second 100 Hz trains in wildtype and S120A slices ($n = 6, 9$ slices). **H**, LTP induction in response to a single 1-second 100 Hz train in slices from wildtype and S120A animals ($n = 6, 7$ slices). **I**, Example traces from voltage-clamp whole-cell recordings conducted in wildtype and S120D hippocampal cultures (DIV 21-24) in the presence of TTX, illustrating miniature excitatory postsynaptic currents (mEPSCs). Scale bar indicates 200 ms, 20 pA. **J**, **K**, Average mEPSC amplitude (J) and inter-event interval (K) from wildtype and S120D mice ($n = 10, 14$ cells). **L**, Cumulative probability plot of all mEPSC amplitudes recorded from wildtype and S120D cultured neurons ($n = 1027, 1485$ events). All graphs represent mean $\pm$ SEM.

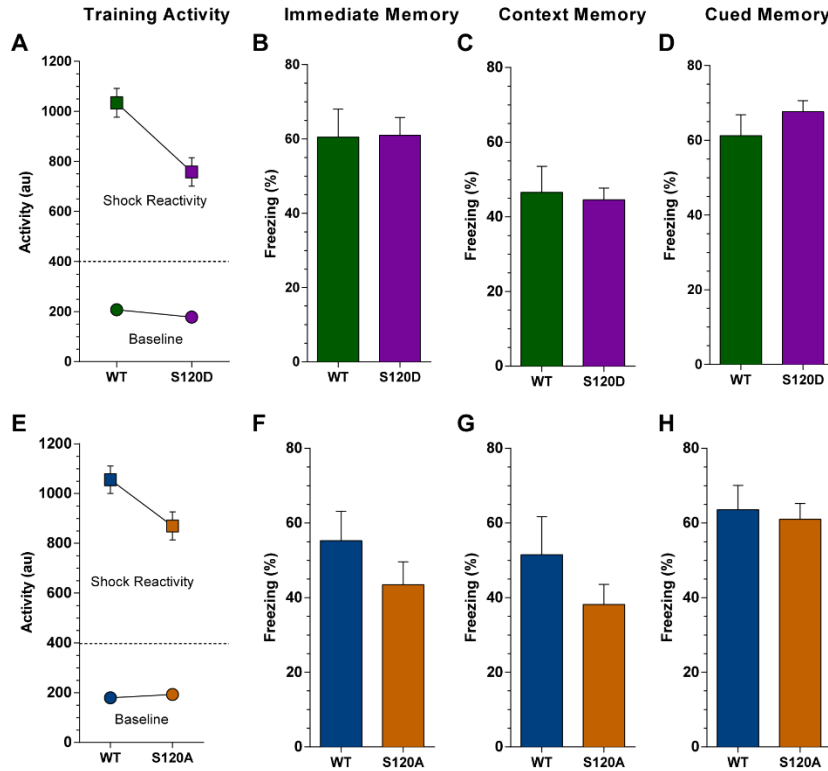


Figure 4-2: Fear memory is not impaired in S120 mutant mice. **A**, Average activity for S120D ($n = 21$) and WT ($n = 13$) mice during the baseline (2 min) and during the shock (2 sec), demonstrating that baseline activity does not differ between S120D and WT mice ($p = 0.27$), though S120D mice exhibited lower shock reactivity (ANOVA, $F(1,34) = 10.48$, $p < 0.005$). **B**, Immediate memory measured as the average percent time freezing during the final 5 min of training, showing no differences between S120D mice and WT mice ($p = 0.95$). **C**, Context memory: 24 h post-training, mice were placed back into the training context and freezing was assessed during the 5 min test, with no significant differences observed between S120D and WT mice ($p = 0.77$). **D**, Cued fear memory: 24 h after the context test, mice were placed in a novel context and presented with three conditioned tones. The average percent time spent freezing during the three tone presentations is depicted, showing no differences between groups ($p = 0.28$). **E**, Average activity for WT ($n = 8$) and S120A ($n = 16$) during baseline (2 min) and shock presentation (2 sec), showing no differences in baseline activity ($p = 0.62$) but decreased shock reactivity in S120A mice (ANOVA, $F(1,22) = 4.33$, $p < 0.05$). **F**, Immediate memory during the final 5 min of training, with no differences between genotypes ($p = 0.27$). **G**, Normal contextual fear memory at 24 h in both WT and S120A mice ($p = 0.73$). **H**, Normal cued fear memory in WT and S120A mice ($p = 0.21$). All graphs represent mean $\pm$ SEM.

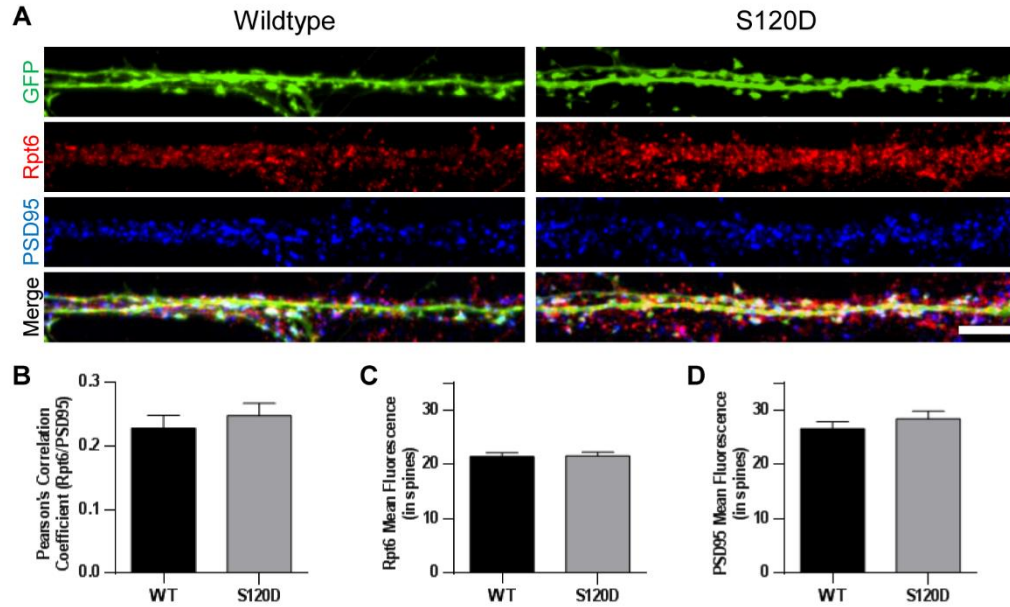


Figure 4-3: S120 phospho-status does not affect Rpt6 localization in dendrites. **A**, Example z-stacked immunofluorescent images of GFP-filled (Sindbis virus) cultured hippocampal neurons (DIV21-24) from wildtype and S120D mice stained with Rpt6 (red) and PSD95 (blue) antibodies. Scale bar depicts 5 μ m. **B**, Pearson's correlation coefficient analysis on Rpt6 and PSD95 signal from dendrites of wildtype and S120D cultured neurons ($p = 0.50$; $n > 18$ dendrites per genotype). **C**, **D**, Quantification of mean Rpt6 (**C**) and PSD95 (**D**) fluorescence within spines in wildtype and S120D neurons, identified via GFP channel ($p = 0.90$; $p = 0.34$; $n > 18$). Graphs depict mean $\pm$ SEM.

Chapter 5:

Conclusions

5.1: Synaptic activity recruits the E3 ubiquitin ligase Nedd4-1

The ubiquitination of several postsynaptic proteins has been reported to regulate their stability and function at synapses (Mabb and Ehlers, 2010). While many proteins likely undergo constitutive ubiquitination, recent evidence suggests that ubiquitination and turnover of synaptic proteins can be regulated in part by synaptic activity (Ehlers, 2003). Ubiquitination can be rapidly reversed by the actions of deubiquitinating enzymes (DUBs). Furthermore, ubiquitination and deubiquitination can be regulated by other substrate-specific post-translational modifications including phosphorylation (Pickart, 2004). Recently, ubiquitination has been shown to be important for the internalization, endocytic sorting, and degradation of AMPARs (Schwarz et al., 2010; Fu et al., 2011; Lin et al., 2011; Lussier et al., 2011). GluA1 and GluA2 subunits are ubiquitinated in response to the agonist AMPA while the NMDAR agonist NMDA does not induce the ubiquitination of these subunits (Schwarz et al., 2010; Lussier et al., 2011). Therefore, while both AMPA and NMDA induce the internalization of AMPARs, ubiquitination appears to be a distinct feature of direct AMPAR activation.

We and others previously reported that Nedd4-1 is a HECT E3 ubiquitin ligase which ubiquitinates GluA1-containing AMPARs (Schwarz et al., 2010; Lin et al., 2011). The precise steps leading up to Nedd4-1-dependent ubiquitination of AMPARs have thus far been unknown. In this regard, we first set out to define the molecular mechanisms which regulate Nedd4-1 function at synapses. In Chapter 2, we provided evidence of rapid redistribution of Nedd4-1 to dendritic spines and synapses in response to AMPAR activation but not in response to NMDAR activation (Fig. 2-1).

Nedd4-1 is rapidly relocalized, on the order of minutes, to dendritic spines in neurons treated with AMPA or glutamate and glycine. This redistribution requires both external calcium entry and ligand binding at the receptor (Fig. 2-1F, G). Hou and colleagues previously reported that Nedd4-1 and polyubiquitin conjugates accumulate at synapses after light-induced activation of AMPARs, however, the mechanism and functional relevance of this phenomenon has until now remained unknown (Hou et al., 2011). Additionally, we have uncovered a critical role for the calcium-sensing C2 domain in Nedd4-1, which has not previously been investigated in neurons. We show that the C2 domain is required for Nedd4-1's ability to traffic to dendritic spines and to reduce surface AMPAR expression (Fig. 2-2). Furthermore, co-application of the AMPAR antagonist CNQX and calcium ionophore ionomycin was capable of mimicking AMPA-induced redistribution of Nedd4-1 to dendritic spines, while either alone was insufficient (Fig. 2-1G). Together, these data suggest that calcium-dependent and ligand-induced conformational changes in Nedd4-1 and AMPARs, respectively, are involved in the rapid redistribution of Nedd4-1 to dendritic spines in response to AMPAR activation.

While this work uncovers a role for calcium in regulating Nedd4-1, it is very likely that the ligase is being regulated through other means, such as posttranslational modifications. Indeed, Nedd4-1 was recently shown to be phosphorylated by the tyrosine kinase c-Src (Persaud et al., 2014). Since many members of the Src family are present in neurons and known to be involved in AMPAR trafficking (Hayashi & Huganir, 2004; Ahmadian et al., 2004), this form of Nedd4-1 regulation could be playing a crucial role in activity-dependent control of this ligase. It is also possible that

non-ubiquitin posttranslational modifications to AMPARs themselves, such as phosphorylation, could affect the ability of Nedd4-1 to ubiquitinate the C-terminal lysines, adding another layer of regulation.

5.2: Nedd4-1 regulates homeostatic downscaling of synaptic strength

Neurons have the capability to sense long-term changes in synaptic activity and scale their synaptic inputs to maintain an excitability set-point (Turrigiano et al., 1998). As Nedd4-1 is capable of altering synaptic strength in response to activity, we hypothesized that chronic changes in activity levels could manipulate the ubiquitin system. Synaptic scaling has been the subject of numerous recent studies seeking to elucidate the molecular processes involved, though upscaling during activity blockade has been investigated far more thoroughly than downscaling. While the ubiquitination and degradation of several synaptic proteins have been shown to be involved in homeostatic scaling (Seeburg et al., 2008; Shin et al., 2012; Mabb et al., 2014), to date, few E3 ubiquitin ligases have been implicated as regulators of downscaling (Fu et al., 2011; Hou et al., 2011). We found that long-term treatment with bicuculline led to a significant increase in total levels of Nedd4-1 (Fig. 2-3; Fig. 5-1). In line with this hypothesis, we observed significantly increased AMPAR ubiquitination in neurons exposed to long-term treatment with bicuculline (Fig. 2-3A). Furthermore, similar treatments promoted the redistribution of Nedd4-1 to synapses (Fig. 2-3D, E). Together, these findings led us to hypothesize that Nedd4-1 could be in part responsible for the decrease in synaptic strength observed in bicuculline-induced downscaling. Indeed, knockdown of Nedd4-1 prevented bicuculline-induced scaling

of both surface AMPARs and synaptic strength (Fig. 2-4), underscoring the importance of this E3 ligase in the dynamics of synaptic scaling (Fig. 5-1).

Our results describe the activity-dependent control of an E3 ubiquitin ligase in the regulation of surface AMPARs and synaptic strength. These results do not preclude the involvement of other enzymes or even other E3 ligases, as it is known that other ligases can directly ubiquitinate AMPARs (Fig. 1-1). Instead, it is likely that a shift in activity levels engages a number of molecular mechanisms at different time scales, all of which contribute to the observed changes in synaptic strength. Dynamic control of synaptic strength involves a number of complementary and competing mechanisms, and AMPARs in particular are subject to a wide variety of rapid post-translational modifications that manipulate surface expression. Interestingly, recent work from our group has also indicated that deubiquitination of AMPARs by the enzyme USP8 is also under control of synaptic activity (Scudder et al., 2014). Our results underscore the critical role of a fine-tunable control of ubiquitination and deubiquitination that allows synapses to adjust to changing activity levels (Fig. 5-1). However, future studies describing the functional and likely dynamic relationship of these modifications (e.g. phosphorylation and ubiquitination) in the regulation of AMPAR trafficking and synaptic plasticity will be of great interest.

5.3: AMPAR ubiquitination as a mechanism for A β -induced synaptic depression

The earliest stages of Alzheimer's disease are thought to be the result of surface glutamate receptor loss, which causes the weakening of excitatory synapses (Hsieh et al., 2006; Chang et al., 2006). While it has been previously theorized that

aberrant endocytosis of AMPARs is driving this weakening, the signaling mechanisms leading to this have been unknown. In Chapter 2, we provided evidence that Nedd4-1 is recruited to synapses following amyloid beta treatment, where the ligase can ubiquitinate surface AMPARs and induce their internalization. Loss of Nedd4-1 prevents the reduction of synaptic strength observed after A β treatments, indicating that it plays a crucial role in disease pathogenesis.

Interestingly, Nedd4-1 appears to operate in response to A β in a similar manner to how it responds to synaptic activity elevation. Activation of synapses, either by agonists or through chronic bicuculline treatments, causes the recruitment of Nedd4-1 and ubiquitination of receptors, leading to reduced synaptic strength at these sites. In a healthy neuron, this provides the neuron with a negative feedback mechanism, allowing it to dynamically alter the strength of inputs. However, in our model of Alzheimer's, it appears that this same homeostatic mechanism is being engaged, resulting in overly weak synapses. The engagement of downscaling mechanisms during synaptic depression in models of Alzheimer's disease has been previously proposed, and this new data supports that earlier hypothesis (Chang et al., 2006).

Our work does not address the mechanistic steps of how Nedd4-1 becomes recruited after A β treatment. This could be the result of neuronal activity alterations amongst treated neurons, potentially involving excessive glutamate release (Talanta et al., 2013) or perhaps altered calcium levels, as has been reported in models of Alzheimer's (Small, 2012). Thus, the calcium responsiveness of Nedd4-1 may be a critical factor. Future studies will be critical in determining how Nedd4-1 becomes

involved in A β -induced synaptic weakening, and potentially how this involvement can be prevented during the development of Alzheimer's.

5.4: Neddylation in excitatory synapse maintenance

Though it is a well-characterized posttranslational modification, the conjugation of NEDD8 (neddylation) has remained largely unstudied in neurons. While a large number of NEDD8 substrates exist in neurons, it is unknown whether this modification plays a crucial role in neuronal function or regulation of synapses. The recent development of a neddylation inhibitor has enabled the investigation of this. In Chapter 3, we presented evidence that NEDD8 conjugation plays a role in the maintenance of mature excitatory synapses. Treatment with the inhibitor led to reduced synaptic strength, surface GluA1 abundance, and dendritic spine width. These effects occurred within 12 hours, indicating that neddylation is playing a role in maintaining the strength of existing synapses.

However, our work does not point to any particular target for NEDD8 conjugation, and a number of targets likely exist at these synapses. Neddylation has known roles in controlling the activity of many E3 ubiquitin ligases, and thus our observed effects could be due to misregulation of ubiquitination of synaptic proteins. Interestingly, a recent paper utilized the neddylation inhibitor and genetic modifications and determined that PSD95, an excitatory synapse scaffolding protein, is itself neddylated. Neddylation of PSD95 has a stabilizing effect on the protein, and inhibition of NEDD8 conjugation leads to reductions in synaptic strength as a result of this. Additionally, inhibition interferes with the development of excitatory synapses.

These results are in line with our own, and suggest that the effects on synapses that we observed could in part be due to loss of PSD95 neddylation. Much remains to be studied regarding the exact role of NEDD8 and how the neuron utilizes this posttranslational modification. Since phosphorylation and ubiquitination can both be induced in response to synaptic cues, it is intriguing to speculate that neddylation could add another layer of activity-dependent regulation. Examining potential roles for NEDD8 conjugation in forms of synaptic plasticity would be of great interest.

5.5: Proteasome Rpt6 phosphorylation and synaptic plasticity

In response to a large body of evidence suggesting that regulation of proteasomes plays a critical role in regulating synaptic function, our lab created the first mouse model with altered proteasome function. Our single-point mutations to Rpt6 S120 into a phospho-mimetic or phospho-dead state resulted in viable mice with no obvious health or behavior issues. Interestingly, despite several studies suggesting that the CaMKII-dependent phosphorylation of this serine is an essential site for regulating proteasomes, we observed no effects on basal synaptic transmission, long-term potentiation, or learning and memory.

We recapitulated the previous findings that the phospho-status of S120 corresponds to altered protease activity in 26S proteasomes, though these changes were relatively small (Gonzales & Patrick, unpublished data). Since proteasome inhibitors such as MG132 and lactacystin essentially abolish proteasome activity, it is unsurprising that our results would not be as robust as those that reported LTP and learning deficits after use of these drugs. However, given that the phospho-status of

S120 has been shown to change in response to activity and during learning paradigms, we had expected to observe some changes in plasticity and learning in our mice.

Our lack of an effect could be due to several factors. One possibility is that this site is simply not important for the types of learning and plasticity that we examined. Perhaps a process like homeostatic plasticity relies on proteasome regulation at this site, requiring long-term changes in synaptic activity. The ability to control proteasome activity could be more important in stress-related conditions or during aging, when careful protein control may be more critical for the survival of the neuron and the organism. Different regions of the brain may also rely on proteasome activity to different extents. Our study focuses mainly on the hippocampus and hippocampus-dependent learning paradigms. Since regions like the striatum have been shown to have high rates of AMPAR synthesis and degradation, perhaps this region would show basal or plasticity-related deficits. Indeed, the S120D mice appear to have abnormal cocaine sensitization, a drug-induced behavioral effect that relies on plasticity in the striatum (Howell, Gonzales et al., in preparation).

Another possible explanation for our results is that the developing nervous system of our mutant mice developed compensatory mechanisms to take over for functions that Rpt6 S120 phosphorylation typically serves. The reduced levels of proteasome activity in S120A neurons, for example, could have induced an increase in lysosomal degradation of synaptic proteins. Such a trade-off has been observed after application of proteasome inhibitors, suggesting that the each degradation system can respond to changes in the other's activity levels (Korolchuk et al., 2010). Thus, future

studies should focus on alternate forms of degradation in the Rpt6 mutant mice in order to determine whether compensation has occurred.

5.6: Relationship between S120 phosphorylation and Rpt6 cellular distribution

Reports have varied regarding whether or not CaMKII-dependent phosphorylation of Rpt6 affects the distribution of proteasomes within neurons. Proteasomes are known to redistribute into dendritic spines in response to synaptic activity, and this requires the presence of CaMKII α , which also undergoes activity-dependent redistribution. However, one study demonstrated that the kinase activity of CaMKII α is dispensable for this, arguing that the kinase functions more as a scaffold for proteasomes. A second report disputes this, observing a change in Rpt6 localization with S120 mutant constructs. The present data, collected with S120 phospho-mimetic and phospho-dead knock-in mice, supports the former model, wherein the direct phosphorylation is unnecessary. S120D neurons displayed no difference in Rpt6 localization within dendrites. However, the present study only focused on basal localization and not on activity-dependent changes. Future studies will reveal whether S120 mutations affect the ability of proteasomes to be recruited to synapses. If such an effect is not observed, then more work will be needed to determine exactly what function the S120 phosphorylation site serves for neuronal proteasomes.

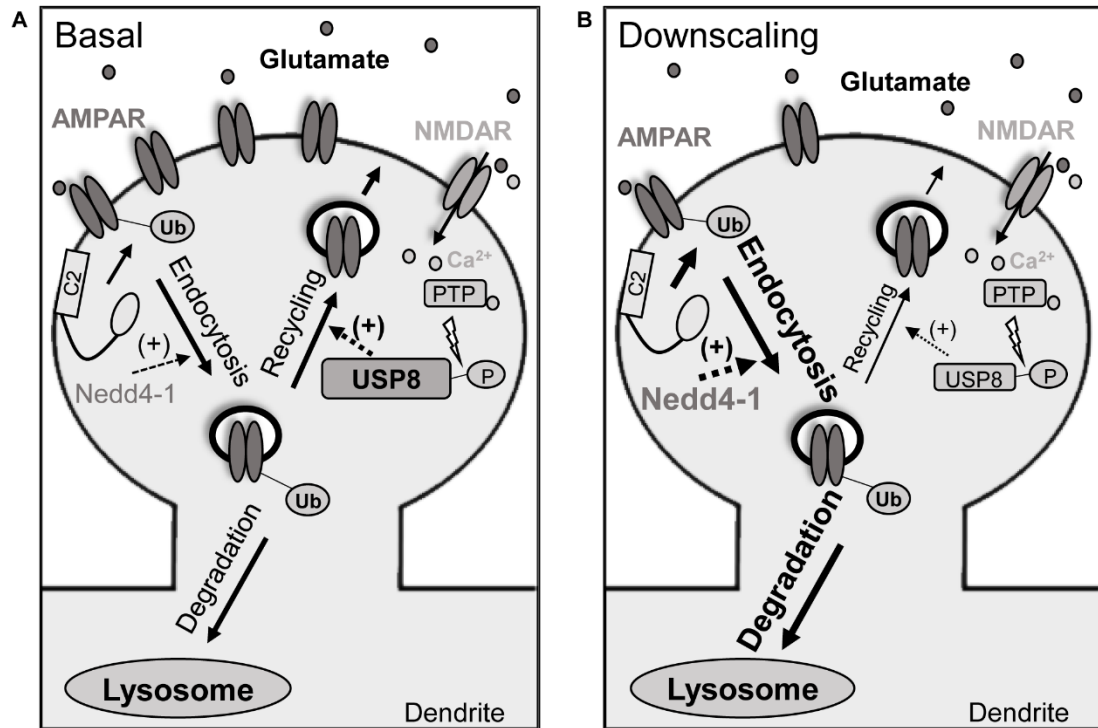


Figure 5-1: Involvement of ubiquitin-dependent AMPAR trafficking in synaptic downscaling. **A**, AMPAR activation recruits Nedd4-1 to dendritic spines through a mechanism dependent on calcium and the C2 domain, leading to AMPAR internalization. NMDAR activation causes activation of USP8 through dephosphorylation by a protein tyrosine phosphatase (PTP), promoting the recycling of internalized AMPARs. **B**, Increased synaptic activity through prolonged bicuculline treatment leads to a decrease in USP8 levels and an increase in and recruitment of Nedd4-1 at synapses, causing a shift in the balance of AMPAR trafficking that overall favors their internalization, resulting in downscaling of synaptic strength.

Chapter 6:

Materials and methods

Antibodies and reagents

Antibodies were purchased from: pAb (C-term) GluA1, pAb surface (N-terminal) GluA1, pAb GluA2/3, and pAb Nedd4-1 (Millipore); mAb PSD-95 and pAb synapsin I (Calbiochem); mAb tubulin (Sigma); mAb actin (Cytoskeleton, Inc.); pAb USP8 (Sigma-Aldrich); mAb ubiquitin (P4D1) (Santa Cruz Biotechnology); mAb GFP (NeuroMab, UC Davis); pAb GFP (Invitrogen); mAb hemagglutinin (HA) (Covance); mAb synaptophysin (Synaptic Systems); pAb NEDD8 (generous gift from Dr. Eric Bennett). Reagents were purchased from: L-Glutamic Acid and Glycine (Fisher); α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-methyl-*D*-aspartate (NMDA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-2-amino-5-phosphonopentanoic acid (APV), tetrodotoxin (TTX), bicuculline, picrotoxin, ionomycin, and BAPTA-AM (Tocris Bioscience); sodium orthovanadate, *N*-ethylmaleimide (NEM), and leupeptin (Sigma); ethylene glycol tetraacetic acid (EGTA) (Calbiochem); lambda protein phosphatase (Millipore); MLN4924 (Active Biochem, Maplewood, NJ); human synthetic A β 1-42 (GenicBio).

Neuronal cultures

Rat dissociated hippocampal or cortical neurons from postnatal day 1 pups of either sex were plated at a density of 45,000 cells/cm² onto poly-D-lysine-coated coverslips, glass bottom 35 mm dishes (hippocampal cultures) (Mattek), or poly-D-lysine-coated 6-well plastic dishes at ~500,000 cells per well (cortical cultures) and were maintained in B27 supplemented Neurobasal media (Invitrogen) until ≥ 14 days

in vitro (DIV), as previously described (Djakovic et al., 2009; Schwarz et al., 2010; Djakovic et al., 2012).

Recombinant DNA, Sindbis, and Lentiviral constructs

Mouse HA-tagged Nedd4-1 in pCDNA3.1(-) was purchased from Addgene DNA repository. This original clone isolated and reported by Kumar et al. in 1992 is considered to be a “near full length” clone of Nedd4-1 which has the minimal C2 domain intact as its size on SDS PAGE is the same as endogenous Nedd4-1 protein (Kumar et al., 1992; Kumar et al., 1997). To create the HA-Nedd4-1 Δ C2 deletion mutant we first cloned an NheI-HA-tag-XbaI sequence into the XbaI site of a double subgenomic Sindbis DNA vector (2Gene Sindbis). PCR deletion mutagenesis was then utilized to clone DNA sequence of mouse Nedd4-1 lacking the first 180 amino acids (Δ C2 deletion) into the XbaI site downstream and in frame with the HA-tag. The HA-Nedd4-1 Δ C2 deletion mutant was then subcloned out of the Sindbis construct into pCDNA3.1(-). Production of recombinant Sindbis virus was performed as previously described (Djakovic et al., 2009). All DNA and viral constructs were verified by sequencing.

Transfections and infections

HEK293T cells, maintained in DMEM plus 10% serum and penicillin/streptomycin, were transfected with Lipofectamine 2000 (Invitrogen) or polyethyleneimine (Polysciences) using recommended protocols. For Sindbis experiments (Nedd4-1 redistribution and Chapter 3 spine analysis), hippocampal or

cortical cultures were infected with Sindbis virion at DIV16-22 and allowed to express for 14–22 h. For RNAi experiments, hippocampal cultures were infected with lentivirus expressing the RNAi constructs for 5-7 days. To fill cells for spine density analysis ($A\beta$ experiments), neurons were infected with Sindbis virion expressing tdTomato for 13 hours or lentivirus expressing GFP for 4 d. Viral titer and transduction efficiency were monitored for all viruses made to ensure equal expression of constructs.

Naturally-secreted and synthetic $A\beta$

Chinese hamster ovary (CHO) cells stably expressing human APP751 with the Val717Phe mutation (7PA2 cells, a generous gift from Dr. Edward Koo) (Podlisny et al., 1995) were cultured in DMEM with 10% fetal bovine serum and penicillin/streptomycin. Neurobasal media with B27 supplement (Invitrogen) was conditioned with confluent 7PA2 cells, cleared of cells by centrifugation, and concentrated using YM-3 Centriprep filters (Millipore). $A\beta_{1-42}$ concentration was assessed by ELISA, using monoclonal antibody MM26-2.1.3 (Mayo Clinic) as capture and HRP-conjugated 6E10 (Covance) antibody as reporter (generously provided by E. Koo). Control conditioned media (CM) was prepared in a similar fashion from CHO cells. For synthetic $A\beta$ experiments, human synthetic $A\beta_{1-42}$ (GenicBio) was dissolved in DMSO then oligomerized in MEM using published protocols (Talanta et al., 2013; generously provided and prepared by S. Lipton and M. Akhtar). Oligomeric stock concentration was determined through dynamic light scattering to be 25 μ M, and final treatment concentration was 250 nM. DMSO alone was used as a control.

Biotinylation of surface AMPARs

To detect surface AMPARs, cultured cortical neurons were rinsed with PBS-MC (10 mM phosphate buffer, 2.7 mM KCl, 137 mM, NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4), placed on ice, rinsed with cold PBS-MC, incubated with 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 15 min at 4°C, and then rinsed again with 0.1% BSA in PBS-MC. Cells were scraped into RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors. An equal amount of protein per sample was incubated with neutravidin-agarose (Pierce) overnight at 4°C. Agarose was then rinsed 3 times, and bound proteins were eluted into SDS sample buffer by boiling. Quantitative Western blots were performed on biotinylated proteins using antibodies against GluA1 (1:1000) or Ned4-1 (1:5000).

Immunoprecipitations

Cultured rat cortical neurons were lysed in precipitation buffer (in mM: 100 NaCl, 10 Na₂HPO₄, 5 EDTA, 5 EGTA) with 1% triton X-100, 0.1% SDS, 25 µM MG-132, 25 mM NEM, and protease inhibitors. Homogenates were cleared by centrifugation at 14,000 rpm at 4°C. For immunoprecipitations (IPs), cleared lysates were incubated with primary antibodies at 4°C for 1.5 h or overnight, after which protein A or protein G agarose beads were added for an additional 1 h (Pierce). Immunoprecipitates were then washed, boiled in sample buffer, resolved on SDS PAGE and probed in western blot analysis. For AMPAR ubiquitination assays, we used anti-GluA2/3 pAb antibodies since this antibody efficiently IP'd both GluA1 and

GluA2 in our lysis buffer conditions which does not disrupt the tetramer receptor complex. In biochemical assays where neuronal cultures were evaluated for ubiquitination status, 100 µg/mL Leupeptin (Millipore) was administered 1 h before treatments unless otherwise specified.

Western blot analysis

Total protein lysates were generated by scraping cells into RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors and incubating for 20 minutes at 4° C. Fractions from adult rat brain tissue were prepared as previously described (Carlin et al., 1980; Cho et al., 1992; Ehlers, 2003). Protein concentration was determined by BCA protein assay (Pierce) and equal protein amounts were loaded. Samples were boiled with sample buffer, resolved on 8% SDS page, and probed with primary and secondary antibodies. Blots were digitized and band intensities were quantified using NIH ImageJ. For quantification of protein levels, band intensities in each condition were normalized to tubulin band mean intensity from the same sample. Statistical significance was determined through unpaired t test using band intensity values from at least three independent experiments.

Immunostaining

Following infections and drug treatments, hippocampal cultured neurons were washed with cold PBS-MC and fixed with a solution containing 4% paraformaldehyde and 4% sucrose for 10 minutes. Cells were then permeabilized with 0.2% Triton X-

100 and 2% BSA in PBS-MC for 20 minutes followed by a 1 hour block in 5% BSA in PBS-MC. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 1 hour at room temperature or overnight at 4° C. Coverslips were mounted onto glass slides for confocal imaging. For surface immunostaining, neurons were fixed, blocked in 5% BSA for 12-20 h at 4° C, and incubated with surface GluA1 antibody for 12-20 h at 4° C. Cells were then permeabilized, blocked, incubated with anti-GFP antibody, and incubated with secondary antibodies.

Confocal microscopy and image analysis

All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nipkon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 $\mu\text{m}/\text{pixel}$ at 1 $\times$), Plan Apochromat 63 $\times$ /1.4 numerical aperture objective, and an argon/krypton 100 mW air-cooled laser for 488/568/647 nm excitations. Maximum projected confocal Z-stacks were analyzed with NIH ImageJ. For Nedd4-1 redistribution experiments, dendrites were straightened and a custom macro was used to run the Find Maxima function on the HA channel through a range of noise tolerance levels ranging from 10 to 255. For further analysis of differences between conditions, a tolerance level between 70 and 100 was chosen for all images. For analysis of differences in GluA1 fluorescence, GluA1 fluorescence within GFP-masked dendrites (from cell-filling Sindbis infection) was quantified and compared across conditions. For spine analysis experiments, dendrites from spiny pyramidal-like neurons were chosen and spines were manually selected. Density, width, and height were quantified using a custom ImageJ macro.

Fluorescence within spines was determined through manual spine tracing and a custom ImageJ macro. Integrated density within spines was compared to total integrated density to determine the fraction of total HA signal present in spines, and values were normalized to control. Statistical significance was determined through unpaired *t* tests or ANOVA with specified post-hoc multiple comparisons test, using Prism (GraphPad).

Electrophysiology

For whole-cell recordings of miniature EPSCs (mEPSCs), dissociated rat hippocampal neurons were bathed at room temperature in a HEPES-buffered saline recording solution containing the following: 119 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 30 mM glucose, 10 mM HEPES (pH 7.2), 1 μ M TTX, and 10 μ M bicuculline. The electrode recording solution contained the following (in mM): 10 CsCl, 105 CsMeSO₃, 0.5 ATP, 0.3 GTP, 10 HEPES, 5 glucose, 2 MgCl₂, and 1 EGTA (pH 7.2). Electrode resistances ranged from 2.5 to 4.5 M Ω and access resistances ranged from 10 to 25 M Ω . Signals were amplified, filtered to 2 or 5 kHz, and digitized at 10 kHz sampling frequency. Holding potential for all traces was -70 mV. mEPSCs were analyzed using ClampFit 10.3 (Molecular Devices). Infected cells were identified through GFP fluorescence. Statistical differences between conditions were analyzed through either unpaired *t* tests (two groups) or by ANOVA and indicated *post hoc* multiple-comparison test (>2 groups), conducted in Prism (GraphPad).

For acute slice experiments (S120 mice), acute hippocampal slices were prepared from 3-8 week old mice with experimenter blind to genotype. Mice were

anesthetized with isofluorane prior to decapitation and brain extraction into ice-cold sucrose-containing ACSF (in mM: 83 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, 72 sucrose, 0.5 CaCl₂, 3.3 MgSO₄). Tissue was sliced coronally into 350 µm slices using a Leica VT1200 vibratome. Slices were recovered in standard ACSF (in mM: 119 NaCl, 5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 11 glucose, 2 CaCl₂, 1 MgSO₄) at 34°C for 30 minutes and at room temperature for at least 30 minutes prior to recordings. Slices were transferred to a submerged recording chamber and perfused with room-temperature (basal transmission experiments) or 30°C (potentiation experiments) oxygenated ACSF (with 100 µM picrotoxin).

A cluster stimulating electrode (FHC) was placed in the stratum radiatum of the CA1 region and current or voltage was injected using an ISO-Flex stimulus isolator (A.M.P.I.) triggered by a Clampex 10.3 (Molecular Devices) or custom IGOR Pro protocol. Recording electrodes were generated from thin-walled capillary tubing (Warner Instruments) using a horizontal pipette puller (P-97 Flaming/Brown Micropipette Puller, Sutter Instruments), resulting in a resistance of 1-3 MΩ, and pipettes were filled with ACSF. The recording electrode was placed 200-300 µm away from the stimulating electrode in the stratum radiatum, along a pathway parallel to the CA1 pyramidal layer. A second stimulating electrode was placed on the opposite side of the recording electrode to serve as a control pathway in LTP experiments. Field responses were recorded using an Axopatch 200B amplifier (Molecular Devices) and digitized using a Digidata 1322 digitizer (Molecular devices), and signals were acquired using Clampex 10.3 or IGOR Pro.

Field excitatory post-synaptic potentials (fEPSPs) were evoked using 100 μ s pulses. Input-output relationships were determined by recording fEPSPs at a variety of stimulus intensities ranging from 30 μ A to 130 μ A and averaging five traces per intensity. Paired pulse facilitation was examined by generating pulses at variable separations (400, 200, 150, 100, and 50 ms) and taking the ratio of the second fEPSP amplitude to the first, with three recordings averaged for each separation. To study long-term potentiation (LTP), a stimulus intensity that produces a response of 0.2 – 0.4 mV was administered once every 15 seconds until a stable baseline of 15 minutes was reached. LTP was induced in one pathway using four sets of 1-second 100 Hz trains separated by 20 seconds and post-LTP responses were recorded from both control and experimental pathways for 40-60 minutes after potentiation.

Behavioral assessment (fear conditioning)

Apparatus: The VideoFreeze system (Med Associates) was utilized to assess freezing behavior, as described previously (Anagnostaras et al., 2010; Carmack et al., 2014). Mice were tested in individual chambers.

Training: Training consisted of a single 10 minute session. Mice were placed in individual chambers and baseline activity was assessed for the first 2 min. Mice then received 3 tone-shock pairings at minutes 2, 3, and 4, which consisted of a 30-sec tone (2.8 kHz, 90 dB) that co-terminated with a 2-sec AC foot shock (0.75 mA, RMS). Immediate memory was assessed by measuring freezing behavior during the last 5 min of training session.

Context Test: Twenty-four hours after the training session, mice were placed into the training context and freezing was measured for 5 minutes to assess fear.

Tone Test: Twenty-four hours after the context test, a 5 min tone test was conducted to assess cued memory. The context was altered by placing white acrylic sheets over the grid floors, using a black plastic triangular insert was used to alter wall shape, and scenting the chamber with a 5% vinegar solution. Near-infrared light was used to create a dark environment. The test consisted of a 2 min baseline followed by the presentation of three 30 sec tones (2.8 kHz, 90 dBA) at minutes 2, 3, and 4, which matched those presented at training.

Data Analysis: Behavioral data were analyzed using multivariate or univariate analyses of variance (ANOVAs). Significance was set at a level of $p \leq 0.05$ (SPSS Statistics Desktop, V22.0).

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