UNIVERSITY OF CALIFORNIA SAN DIEGO

Establishing New Genetic and Pharmacological Based Models to Investigate the Role of Nedd4-1 in AMPA Receptor Trafficking

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

in

Neuroscience

by

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The dissertation of Nicole C. Hoffner is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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DEDICATION

I dedicate this publication to my grandpa John. J. “Yankee” Hoffner, whose actions provided me with an opportunity for higher education.

I reflect on my entire academic journey in his memory.

EPIGRAPH

If a solution fails to appear ... and yet we feel success is just around the corner, try resting for a while. ... Like the early morning frost, this intellectual refreshment withers the parasitic and nasty vegetation that smothers the good seed. Bursting forth at last is the flower of truth.

Santiago Ramón y Cajal

Growing in size and complexity
living things
masses of atom
DNA, protein
dancing a pattern ever more intricate.

Out of the cradle
onto dry land
here it is
standing:
atoms with consciousness;
matter with curiosity.

Stands at the sea,
wonders at wondering: I
a universe of atoms
an atom in the universe.

Richard P. Feynman

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Chapter 3 includes coauthored material. Hoffner, Nicole; Gilmore, Steven; Dozier, Lara; Patrick, Gentry. Chapter 3, Potent Small Molecule Inhibitor of Nedd4-1, 1-benzyl-indole-3-carbinol, in Neurons. The thesis author was the primary investigator and author of this material.

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ABSTRACT OF THE DISSERTATION

Establishing New Genetic and Pharmacological Based Models to Investigate the Role of Nedd4-1 in AMPA Receptor Trafficking

by

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The purpose of this dissertation research is to advance our understanding of the molecular mechanisms underlying synaptic plasticity and neuronal communication.

Neurotransmitter receptors mediate signal transduction between neurons, and signaling mechanisms are dependent on the identity and quantity of receptors expressed on the surface of the neurons. Surface receptor expression – as a result of e.g. synthesis, trafficking, removal – must therefore be carefully regulated to maintain proper communication. These receptors undergo protein modifications that choreograph their delivery, stabilization, or removal from the membrane surface. One such modifying enzyme is

called Nedd4-1, Neural precursor cell-expressed, developmentally down-regulated protein 4-1, an E3 ubiquitin ligase, which has been shown to modify the GluA1 subunit of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPARs) with a ubiquitin molecule. While the implications that Nedd4-1 is involved in AMPAR-dependent forms of synaptic plasticity remain unambiguous, we aim to assess which of many forms of synaptic plasticity Nedd4-1 may be involved in and thus its role in the regulation of synaptic plasticity.

In the following dissertation, Chapter II describes our efforts in characterizing a neuronal cell culture model. In this chapter, I specifically demonstrate that AAV-cre robustly abolishes Nedd4-1 protein in cultures derived from Nedd4-1 floxed mice. Importantly, unlike the original embryonically lethal *in vivo* knockout models or *in vitro* cultures with significant dendritic abnormalities, we find that in our neuronal cultures, knockout cells are morphologically healthy and amenable to standard biochemical, imaging, and physiology experiments, greatly facilitating our capacity to interrogate Nedd4-1 function in AMPAR trafficking and in health and disease.

Finally, since Nedd4-1 is known to participate in the molecular mechanisms underlying synaptic plasticity, we aimed to delineate the specific forms of receptor internalization Nedd4-1 may be involved in. Chapter III provides a brief validation of a small molecule inhibitor of Nedd4-1, while Chapter IV expounds on our work to study two forms of synaptic plasticity paradigms. We found evidence that Nedd4-1 may be involved in AMPAR internalization following glycine-induced long-term potentiation, but not in NMDA-induced chemical long-term depression. These results help refine our understanding of Nedd4-1 function in neurons and point to new directions for future investigations.

Chapter I

Introduction

A. Protein homeostasis underlies neuronal health and function

Protein homeostasis, or proteostasis, is an overarching cellular operation in which a variety of diverse molecular processes work together to maintain proper control over the expression and regulation of all proteins present within a cell. This includes the initiation of protein synthesis when new proteins are required, and protein degradation when proteins are mutated, misfolded, aggregated or no longer needed. Aging, as well as the dysregulation of these homeostatic regulatory processes leads to a decline in the cell's functional capacities and overall health, as interruptions to synthesis, recycling, degradation, etc. leads to disease states (Hipp et al., 2019). Therefore, maintaining protein homeostasis is critical for the ongoing health and function of cells.

Additionally, the complete profile of proteins a cell expresses, known as its proteome, largely determines the cell's identity and its function. The precise control of protein synthesis and degradation at specific and different locations in particular allows the cells to diversify the protein composition at subcellular compartments, granting a variety of diverse functions within a single cell. This becomes increasingly apparent when investigating highly polarized cell types, such as neurons, which have distinct proteomic profiles in different cellular regions, i.e. the soma, dendrites, and axons. These distinct region-specific proteomic profiles give rise to the cells ability to, for example, synthesize proteins in the soma, while receiving signals at their dendrites, and at the same time sending signals via axons.

In the brain, one example of how proteostasis contributes to cell function is through its regulation of all the proteins required for neuronal communication. Neurons communicate at molecular structures called synapses, and three key structural components present at a chemical synapse include (1) the presynaptic terminal, where a signal is generated and released from one cell to another, (2) the intermediate space between the two cells through which the signal travels, known as the synaptic cleft, and (3) the site on the secondary cell where these signals are received, known as the postsynaptic terminal (Figure 1.1). The structure of the presynaptic and postsynaptic terminals is dictated by the proteins expressed in the membrane, giving rise to their electrical and signaling properties, and therefore the opportunities for these neurons to interact through synaptic signaling mechanisms. When proteostasis is disturbed at synapses, it can lead to aberrant expression of signaling receptors, causing significant impairments to neural communication.

In order to function properly, neuronal synapses require the appropriate expression of signaling receptors at the surface of the cell, as dynamically required for short term (Hebbian-type) and long-term (homeostatic type) changes to synaptic strength, known as synaptic plasticity (Collingridge et al., 2004; Malinow & Malenka, 2002; Turrigiano, 2000). These changes in synaptic input activity – e.g. increased or decreased signal frequency or binding of specific neurotransmitters – engage intracellular proteostatic processes to control receptor trafficking and surface expression. Receptor trafficking to the membrane involves a concerted cycle of exocytosis (delivery of the receptor to the membrane through extravasation of the transport vesicle) and endocytosis (internalization of the surface receptor via membrane invagination and formation of an intracellular transport vesicle). The specific mechanisms

controlling exocytosis (insertion) and endocytosis (removal) of these receptors involve protein post-translational modifications (PTMs) such as acetylation, glycosylation, methylation, phosphorylation, and ubiquitination, to name a few. These modifications can affect the expression, localization, stability, and binding partners of the modified proteins, enabling a wide degree of flexibility and control over cellular functions. Since different PTMs engage separate proteostatic processes, understanding how the dynamic interplay between PTM pathways regulates neurotransmitter receptor expression is critical to understanding cellular biology and proteostatic dysfunction and disease.

The Ubiquitin Proteosome System (UPS) is one proteostatic mechanism that controls protein sorting and degradation, under homeostatic conditions as well as in response to disease states (Figure 1.2). The ubiquitination of proteins requires the attachment, or ligation, of a ubiquitin molecule to specific amino acids on the target protein by a ubiquitinating enzyme, or ligase. One such ligase that has been shown to contribute significantly to receptor internalization, sorting, and disease is Neural precursor cell-expressed, developmentally down-regulated protein 4-1 (Nedd4-1). Nedd4-1 ubiquitinates synaptic proteins that control neuronal communication, and dysregulation of Nedd4-1 is implicated in many diseases such as cancer and neurodegenerative disease (Huang et al., 2019; Schmidt et al., 2021). For example, our lab has implicated Nedd4-1 in Alzheimer's Disease pathogenesis, finding that Nedd4-1 is required for Aß-induced reductions in surface expression of important neurotransmitter receptors (Rodrigues et al., 2016). Additionally, Nedd4-1 protein levels were found to be elevated in *post-mortem* brain tissue of Alzheimer's Disease patients (Kwak et al.,

2012). This provides strong evidence that ubiquitination is an important regulator of proteostasis and a necessary component of cell health maintenance.

B. Nedd4-1 is involved in proteostasis

Nedd4-1 structure and function

Nedd4-1 is a member of the Nedd4 subfamily of E3 ubiquitin ligases containing a HECT (Homologous to EA6P C-Terminus) domain. Similarly to the other 8 HECT E3 ligases in the Nedd4 family, Nedd4-1 shares conserved structural similarities including a lipid-binding C2 domain for membrane targeting, four WW (tryptophan-tryptophan) domains that confer substrate specificity, and a C-terminal HECT domain that catalyzes the ubiquitin ligation (Figure 1.3) (Ingham et al., 2004).

Nedd4-1 is widely expressed in many tissues and has a large variety of protein targets, implicating it in many different mechanisms underlying cell health, in addition to its regulation of surface protein internalization. In whole, Nedd4-1 is known to be involved in the regulation of developmental processes, degeneration, and disease states (Huang et al., 2019), and early Nedd4-1 deficiencies can lead to poor organ development and survival, gross motor deficits, and gait abnormalities (Camera et al., 2014; Cao et al., 2008; Chopra et al., 2018). Effective model systems are necessary in order to tease apart the varied roles and functions of Nedd4-1. Genetic models and small molecule inhibitors of the catalytic HECT domain allow us to experimentally interrogate Nedd4-1 function in the context of synaptic plasticity, and provide additional opportunities to explore Nedd4-1 as a potential target for therapeutic applications (Nguyen et al., 2010; Quirit et al., 2017).

Nedd4-1 regulation of AMPARs and synaptic plasticity

A notable target of Nedd4-1 is the AMPA-type glutamatergic neurotransmitter receptor, AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), a ligand-gated ion channel that mediates neuronal communication. Past work from our lab and others have demonstrated that Nedd4-1 binds to and ubiquitinates AMPARs, which contributes to their subsequent internalization (Schwarz et al., 2010; Scudder et al., 2014). Whether or not AMPARs ubiquitination by Nedd4-1 is the causal event to drive internalization is unclear, however this reveals very important ubiquitination-based underpinnings of neuronal communication. Synaptic signaling provides cues to drive intracellular sorting of AMPARs (leading to and) following their internalization, and we aim to elucidate how the Nedd4-1 dependent ubiquitin pathway is activated by such synaptic activity. For instance, as we've seen in our previous work, stimulation of AMPARs, via AMPA agonist activation, leads to the ubiquitination and internalization of AMPARs and their targeting to late endosomes and lysosomes for degradation. In contrast, the current understanding of NMDAR-mediated AMPAR trafficking is that it relies on the phosphorylation state of the GluA1 subunit of AMPARs, but there is evidence of dynamic interplay between the two PTM signaling mechanisms of ubiquitination and phosphorylation under these contexts. As of yet, it is not fully understood how crosstalk between ubiquitination and other PTMs such as phosphorylation, under these synaptic cues, contributes to the complexity of AMPAR trafficking and is therefore of great import to the field.

C. AMPA receptors mediate neuronal signaling

AMPARs are a subclass of glutamatergic surface receptors

Glutamatergic neurotransmitter receptors are a class of transmembrane, ligand-gated ion channels that mediate the majority of excitatory synaptic transmission. Subclasses of glutamate receptors include kainate, NMDA, and AMPA receptors, defined by their sensitivity to these kainite, NMDA, and AMPA compounds. AMPARs are ligand-gated ion channels with a fast turnover rate compared to the other glutamatergic receptors (Dörrbaum et al., 2020). They mediate the fast-synaptic signaling between cells by permitting sodium influx into the cell upon agonist activation, thereby leading to depolarization, action potentials, and continued signal propagation through the network. They are therefore critical and necessary components of neuronal activity and neural communication.

AMPAR structure and characteristics

AMPARs are heterotetrametric channels, consisting of 2 homomeric subunit pairs, in combinations formed from the dimerized subunits GluA1, GluA2, GluA3, and GluA4. The most common subunit combinations in neurons are GluA1/GluA2 and GluA2/GluA3. Both of these receptor conformations contain the GluA2 subunit, which has a Q/R edit site within the pore domain. This edit confers an impermeability of the channel's pore to large ions, ensuring that sodium (Na^{+}) enters the cell but not larger ions such as magnesium (Mg^{2+}) or calcium (Ca^{2+}), for example. Importantly, a unique population of AMPARs lack this GluA2 subunit and exist in a homomeric GluA1/GluA1 combination. Since these receptors lack the

impermeability-conferring edit site, they permit the large ion calcium into the cell. This is notable because calcium is a critical molecule for many intracellular functions; it is involved in neurotransmitter release, signal transduction via signaling cascades, and as a cofactor in many enzymatic reactions. These calcium-permeable AMPARs (CP-AMPARs) therefore play a unique role in synaptic plasticity, diverging from the canonical, strictly-sodium-permitting AMPARs and calcium-permitting but voltage-gated NMDARs. The regulation of GluA1 synthesis and degradation dynamics under synaptic activity paradigms appear to be uniquely fast, providing further evidence that homomeric GluA1 receptors may exhibit a separate and specialized function in synaptic plasticity (Dörrbaum et al., 2020).

Receptor trafficking via post-translational modifications

In comparison to the highly conserved ligand binding domain (~90% identical) and transmembrane region/ion pore domain (~90% identical) of AMPARs, the intracellular C-terminal tail of GluA subunits exhibits roughly 24-78% identical sequences between the subunit types (Diering & Huganir, 2018). It is in this tail region of the receptor that we find the regulatory PTM sites that control protein localization and degradative fate. AMPARs undergo a variety of PTMs on this C-terminal tail: nitrosylation, O-glycnacylation, palmitoylation, phosphorylation, and ubiquitination. The GluA1 and GluA2 subunits are known to express ubiquitinatable lysine residues, however, Nedd4-1 has been shown to interact with GluA1 specifically and not GluA2. This points to an interesting divergence in the maintenance of AMPARs by Nedd4-1, and suggestive of Nedd4-1 playing a variety of roles in synaptic plasticity, especially as it relates to the homomeric GluA1, CP-AMPAR channel regulation and functional consequences of its expression.

Historically, the function of ubiquitination modification in AMPAR trafficking has been contested. Different research groups sought to reveal whether ubiquitination occurs at the surface prior to internalization, or whether ubiquitin conjugation occurs after the initiation of receptor internalization, serving to direct receptors toward degradation and prevent recycling. While there is competing evidence as to whether ubiquitination occurs before or after receptor internalization, it is undeniable that internalization is mediated by a complex interplay between ubiquitin and non-ubiquitin signaling pathways. Evidence suggests that the mechanisms regulating activity-dependent receptor trafficking respond to finely tuned, context specific, cues, and it will be interesting to find how Nedd4-1 dependent AMPAR ubiquitination interfaces with other post-translational modification based signaling pathways to dynamically control receptor expression.

D. Establishing a primary culture model of Nedd4-1 knockout

Chapter II details the process of developing and characterizing a cell culture based experimental framework to model Nedd4-1 knockout. This chapter outlines the strategy we employed to eliminate endogenous Nedd4-1 *in vitro*. Briefly, we generated dissociated primary neuronal cultures from mice that homozygously expressed a Nedd4-1 allele flanked by LoxP sites, which is recombined out in the presence of cre recombinase. Importantly, we drove recombination only after critical developmental stages, to circumvent phenotypes associated with the developmental defects that might impair our ability to assess Nedd4-1's role in mature neurons. Following the complete ablation of Nedd4-1 protein, these cultures serve as a knockout model of Nedd4-1. Here, we establish that in axon-containing, pyramidal-

type neurons, AAV-cre viral transduction indeed leads to a significant reduction of Nedd4-1 protein. Because knocking out Nedd4-1 in this way does not significantly impact dendritic branching, spine formation, and synaptic signaling, moving forward we can reliably assess the impact of Nedd4-1 knockout on endogenous receptor trafficking *in vitro* using these cultures.

E. Validation of 1-benzyl-indole-3-carbinol

Chapter III describes our validation of the small-molecule inhibitor of Nedd4-1, 1-benzyl indole-3-carbinol (1BI3C), an analogue of the naturally occurring I3C. We demonstrate that 1BI3C blocks auto-ubiquitination of Nedd4-1 and that cell health is not compromised when added to neuronal culture media for up to 24 hours. These results indicate that 1BI3C can be used to inhibit Nedd4-1 function on briefer timescales than the knockout, providing a complementary tool to assess Nedd4-1's role in synaptic plasticity. Interestingly, the naturally occurring form of I3C has been used in oncology research with the potential for therapeutic interventions, and so this work further adds to the discourse of Nedd4-1 function in disease and the therapeutic opportunities arising from inhibition of Nedd4-1 activity.

F. Nedd4-1 and synaptic plasticity

Finally, in Chapter IV we begin to apply our validated methods of Nedd4-1 knockout (Chapter II) and Nedd4-1 inhibition by 1BI3C (Chapter III) to understanding Nedd4-1's function in synaptic plasticity. Our results provide evidence that Nedd4-1 may play two separate roles in receptor trafficking, depending on the cue that initiates AMPAR internalization. Here, we have focused on the activation-induced internalization of AMPARs,

but much work is left to do to uncover how Nedd4-1 engages intracellular sorting pathways after internalization, independent of synaptic activity and regulation of surface receptors.

G. Figures

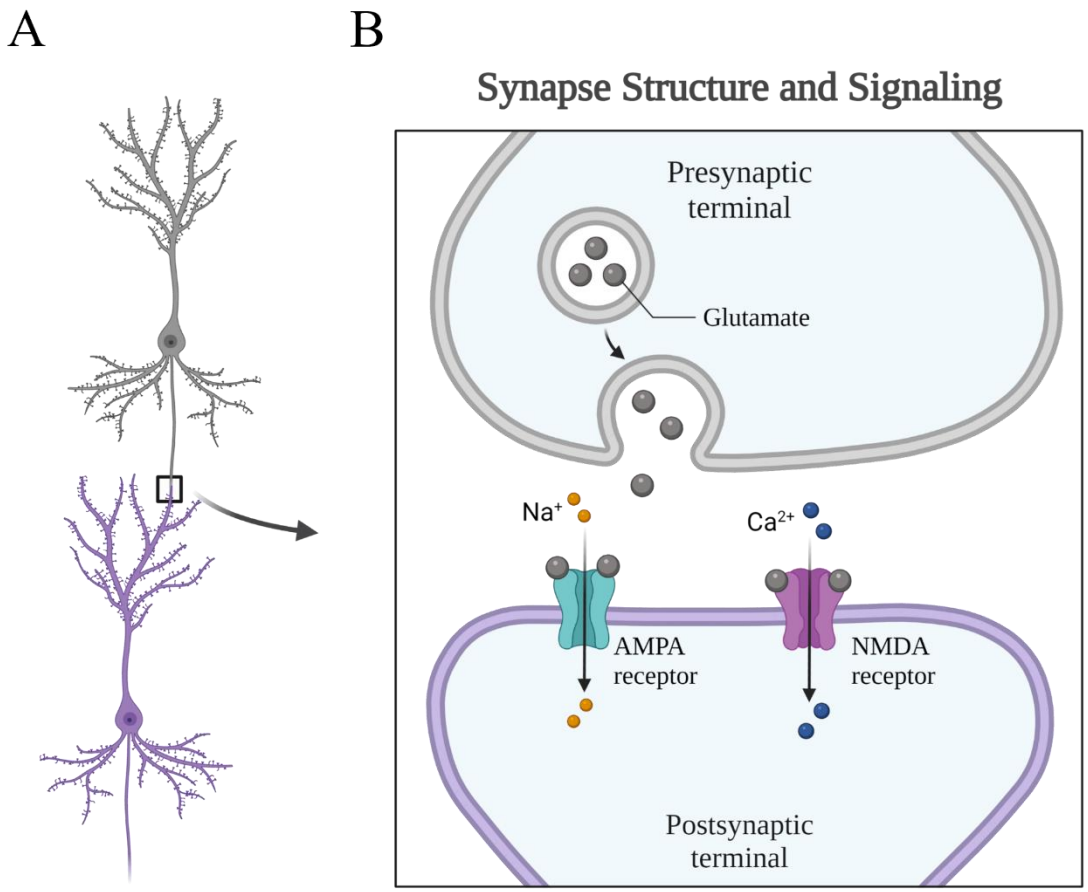

Figure 1.1. Schematic representation of a neuronal synapse. (**A**) Two neurons in close proximity have the opportunity to exchange information at (**B**) chemical synapses from which neurotransmitters such as glutamate are released from the presynaptic terminal and bind to neurotransmitter receptors located in the postsynaptic terminal. This binding of transmitter to receptor contributes to the activation of the postsynaptic cell and initiation of downstream molecular processes. *Made with Biorender*.

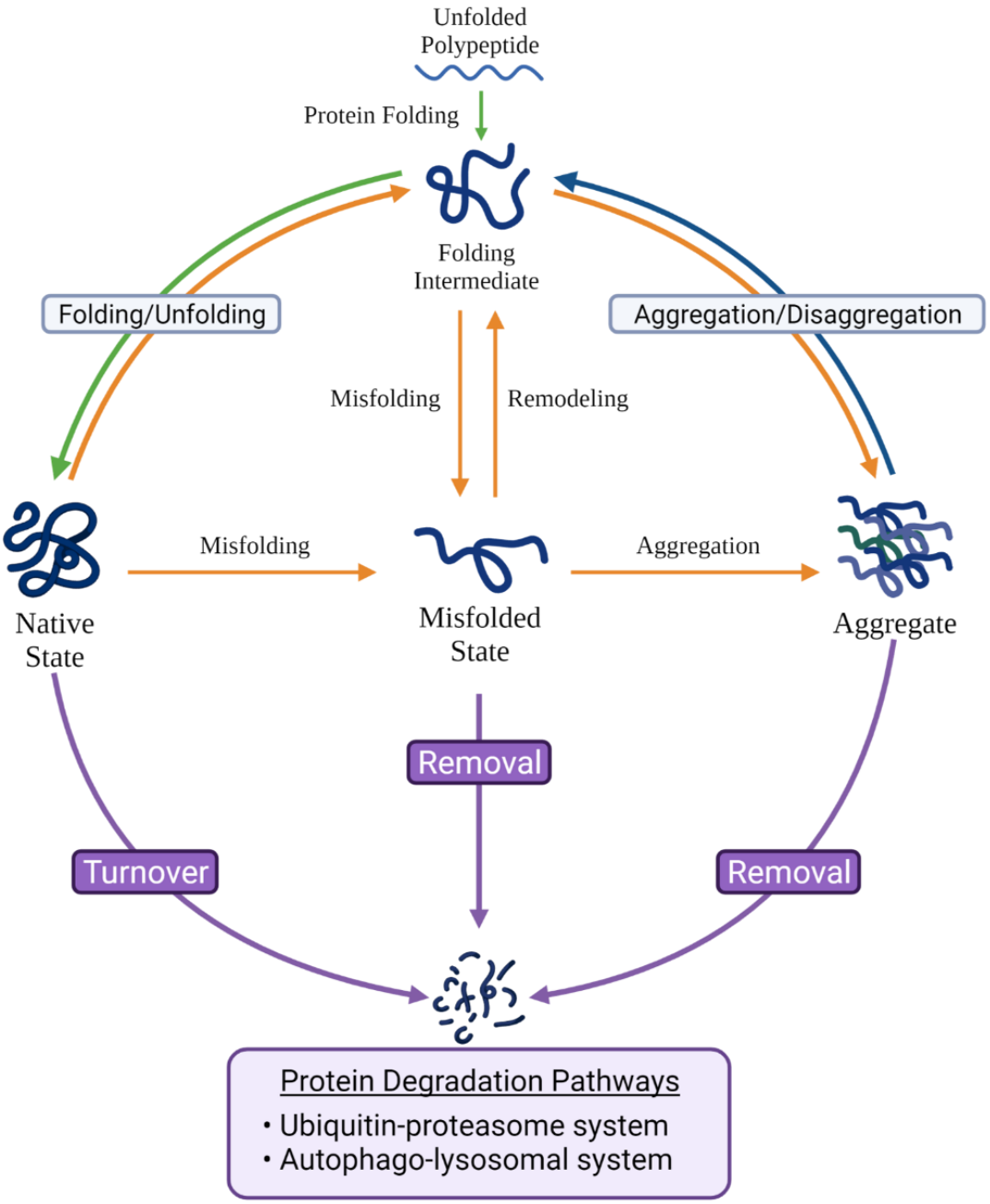

Figure 1.2. Nedd4-1 function in proteostasis. This schematic gives a broad overview of the opposing molecular mechanisms underlying proteostasis. Once nascent polypeptides are newly synthesized (top of illustration), they must be folded into the correct structure in order to function properly. Proteins are removed for degradation for multiple reasons including (1) homeostasis turnover of still-functional proteins, (2) removal of misfolded and non-operational proteins, and (3) removal of potentially harmful aggregates. *Made with Biorender*.

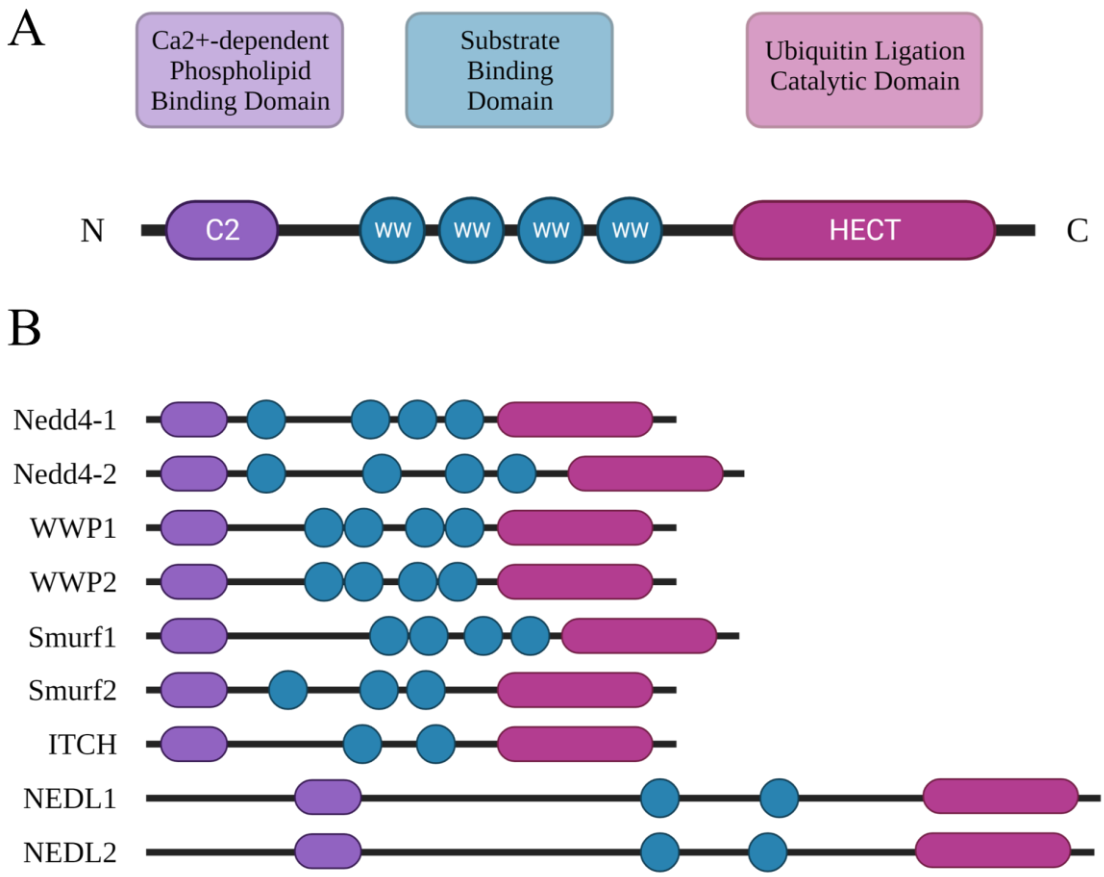

Figure 1.3. Protein structure of Nedd4-1. (**A**) E3 ubiquitin ligases of the Nedd4 family contain 3 conserved functional motifs: an N-terminal C2 phospholipid binding domain that targets the enzyme to intracellular membranes in a calcium-dependent manner (purple); 2-4 tryptophan-tryptophan (WW) domains that bind to PY motifs on substrates and therefore confer substrate specificity (blue); and a conserved HECT domain that catalyzes the enzymatic rection to transfer a ubiquitin molecule from the E3 ligase to the target substrate (pink). (**B**) Comparative structural domains of the 9 human Nedd4-family of E3 ligases. Adapted from Ingham et al., 2004. *Made with Biorender*.

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Chapter II

Nedd4-1 Conditional Knockout Characterization in Mature Primary Neuronal Cultures

A. Abstract

Neural precursor cell-expressed, developmentally down-regulated protein 4-1 (Nedd4-1) is an essential enzyme that contributes to the molecular signaling pathways that regulate protein sorting and degradation. It catalyzes a protein modification called ubiquitination, which is necessary for protein homeostasis and maintenance of cell health in tissues. Studying Nedd4-1 function *in vivo* has historically been difficult to accomplish, as total knockout of Nedd4-1 leads to significant developmental deficits and lethality. This embryonic lethality hinders our ability to study the later-stage, non-developmental functions of Nedd4-1 using genetic knockouts and highlights the need for novel approaches to manipulate Nedd4-1 in ways that maintain cell health and limits off-target effects. To circumvent these early developmental defects and assess Nedd4-1 function in mature neurons, we validated an *in vitro* AAV-based conditional knockout model, characterizing dendrite arborization, dendritic spine properties, synapse expression, and synaptic activity. We found that axon-containing, pyramidal-type neurons exhibited arborization, spine, synapse, and activity properties similar to that of wild type neurons at 14 days *in vitro* (DIV14). These cultures serve as a conditional knockout model of Nedd4-1 in which we can reliably assess the impact of Nedd4-1 knockout on neuronal mechanisms of endogenous receptor trafficking *in vitro*.

B. Introduction

Nedd4-1 (Neural precursor cell-expressed, developmentally down-regulated protein 4-1) is a highly conserved and essential gene coding for a family of HECT E3 ubiquitin ligase

enzymes that target protein substrates for a post-translational modification called ubiquitination.

Throughout the body, Nedd4-1 supports the critical functions underlying health. It is known to be involved in the early regulation of developmental processes, degeneration, and disease states (X. Huang et al., 2019; Schmidt et al., 2021). Nedd4-1 deficiencies can lead to poor organ development, gross motor deficits, and gait abnormalities (Camera et al., 2014; Cao et al., 2008). It is also involved in epidermal homeostasis and wound repair (Yan et al., 2022)

In neurons, Nedd4-1 plays a large variety of roles in both neurodevelopment and neurodegeneration (Haouari et al., 2022). Nedd4-1 plays a role in cell proliferation and autophagy (Li et al., 2015), and through inhibition of kinase function regulates neurite growth and synaptic formation (Drinjakovic et al., 2010; Kawabe et al., 2010; Lin et al., 2011). In neurodegenerative diseases, previous groups have found that Nedd4-1 plays a role in the ubiquitin-mediated protein regulation of α-Synuclein diseases (Davies et al., 2014; Kim et al., 2016; Sugeno et al., 2014; Tofaris et al., 2011), Parkinson's disease (Canal et al., 2016), Alzheimer's disease (Alrosan et al., 2019; Rodrigues et al., 2016), and skeletal muscle atrophy (Nagpal et al., 2012). Further, Kwak et al. demonstrated that Nedd4-1 protein levels were elevated in *post-mortem* brain tissue of Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, and amyotrophic lateral sclerosis (ALS) patients (Kwak et al., 2012).

Nedd4-1 has also been shown to regulate an abundance of disease mechanisms and to contribute to signaling pathways in a wide variety of cancer types (Wang et al., 2022; Zou et

al., 2015). For instance, through its regulation of the tumor suppressor gene phosphatase and tensin homolog (PTEN), Nedd4-1 has been shown to be involved in lung cancer (Amodio et al., 2010; Booth et al., 2018; Shao et al., 2018; Song et al., 2018; Sun et al., 2017), breast cancer (Brauer & Tyner, 2009; Chen et al., 2016; Shen et al., 2019), hepatocellular carcinoma (Hang et al., 2016; Z. J. Huang et al., 2017), prostate cancer (Xu et al., 2003), pituitary adenomas (Zhang et al., 2017), pancreatic ductal adenocarcinoma (Weng et al., 2017), bladder cancer (Wen et al., 2017), and melanoma (Aronchik et al., 2014). Independent of its regulation of PTEN or as yet unknown functions, Nedd4-1 has also been implicated in colorectal cancer (Eide et al., 2013) and gastric cancer (Yang et al., 2012). This provides compelling evidence for the need to understand Nedd4-1's function in a variety of biological contexts.

The context we are explicitly interested in involves a substrate that Nedd4-1 ubiquitinates in the brain, AMPA-type glutamatergic neurotransmitter receptor (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, AMPARs). AMPARs regulate a large majority of synaptic signaling in the brain, and previous work from our lab and others have demonstrated that the regulation of mammalian AMPAR surface expression is mediated by Nedd4-1 dependent ubiquitination. AMPA receptor ubiquitination promotes receptor internalization and subsequent degradation (Lin et al., 2011; Lussier et al., 2011; Schwarz et al., 2010). We found that this Nedd4-1 dependent modification responds in an activity-dependent manner with a high degree of specificity from upstream activation signals and to downstream targets (Schwarz et al., 2010). It will be important in the future to adapt new tools to further investigate Nedd4-1 function in activity-dependent receptor internalization.

The developmental defects that arise due to early embryonic knockout lethality make it impossible to study basic questions of mature brain function and disease using the global knockout method (Cao et al., 2008; Liu et al., 2009). Instead, conditional knockout using the cre-Lox system provides greater spaciotemporal flexibility of knockout, and so we adapted this approach. We express cre recombinase using AAV transduction after 7 days *in vitro* (DIV7) in order to circumvent developmental effects. Using fluorescent imaging, western blotting, and electrophysiology in these cultured neurons, we demonstrate that AAV-cre infection at DIV7 significantly reduces Nedd4-1 protein expression with minimal impact on dendritic development or synapse formation and signaling, providing as a reliable framework to test Nedd4-1 function in mature neurons.

C. Materials and Methods

Mouse Primary Cortical Cultures and AAV infection: Cortical tissue (outer cortex and hippocampus) were obtained from P1 homozygous Nedd4-1 floxed mice that recombines out the Nedd4-1 allele only with the addition of cre. Transgenic animals were kindly provided by Dr. Hiroshi Kawabe (Max Planck Institute). Cortex and hippocampi tissues were dissected from the brains of either sex pups in ice-cold dissection media and transferred to a 37° C water bath. Tissues were enzymatically digested using papain (Worthington Biochemical Co. LS003127) solution and subsequently rinsed in plating media (PM) and transferred to an enzyme-inactivating solution. The cells were then triturated in PM, centrifuged, resuspended, and plated on poly-D-lysine (Fisher Scientific 354210) coated coverslips or poly-D-lysine 6 well plastic dishes. Cultures were maintained in B27-supplemented Neurobasal media

(Invitrogen) at 37° C, 5% CO_2, and 95% relative humidity until 14–21 days. AAV1-Hsyn-dsRed (1.61E+11 titer units/mL) and AAV2-CAG-mCherry-p2A-Cre 2.76E+12 titer units/mL (Salk GT3 Core) were diluted 1:10 and 1:50, respectively, and added directly to the culture media between DIV 7–10 and incubated for 7–14 days of infection.

Western blot analysis. Total protein lysates were generated by scraping cells into RIPA buffer (50 mM Tric-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors and incubated for 20 minutes, rotating at 4°C. Protein concentration was determined by BCA protein assay (Fisher Scientific 23225) and was normalize prior to sample buffer addition. Samples were boiled with sample buffer, resolved on 4-12%, Tris-Glycine gel (Invitrogen Novex Mini Protein Gel XP04122), transferred to nitrocellulose paper, and probed with primary antibodies. Blots were digitized and band intensities were quantified using Fiji (ImageJ). Band intensities were normalized to the band mean intensity of tubulin from each respective sample.

Immunocytochemistry: DIV14-18 neurons were washed with cold PBS-MC (phosphate buffered saline + magnesium and calcium) 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. Tables 1 and 2 list the primary and secondary antibodies used for immunocytochemistry, respectively. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 1 hour at room temperature. Coverslips were mounted onto glass slides for confocal imaging.

Primary and secondary antibodies. Cre Recombinase (D7L7L) Rb 1:2000CST (15036); RFP Rat 1:1000 Chromotek 5F8-20; Nedd4-1 WW2 domain Rb 1:1000 EMD Millipore 07-049; Map2 Ck 1:20000 Abcam Inc. ab5392; PSD95 Ms 1:1000 EMD Millipore CP35; Bassoon Ms 1:2000 Enzo VAM-PS003; Ms-488 Goat 1:1000 Life Technologies A11001; Rb-488 Goat 1:1000 Life Technologies A11034; Rt-488 Goat 1:1000 Fisher Scientific A11006; Ck-647 Goat 1:1000 Fisher Scientific A21449.

Confocal microscopy. All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nikon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 m/pixel at 1X), Plan Apochromat 40X/1.0 or 63X/1.0 numerical aperture objective, and an argon/krypton 100mW air-cooled laser for 488/568/647nm excitations. Maximum projected confocal Z-stacks were analyzed with ImageJ. Statistical significance was determined through unpaired t tests or ANOVA with specified post hoc multiple-comparisons test using Prism software (GraphPad).

Dendrite characterization. Dissociated cortical neurons were infected for 7-9 days with the AAV viruses outlined above and imaged live between DIV14 and DIV16. Axon-containing pyramidal-shaped neurons were selected for analysis, and 20 images were acquired for each cell at 0.5um intervals for a 10um *z*-plane stack. 8-bit *z*-projections were analyzed using the Fiji (ImageJ) plugin Simple Neurite Tracer (SNT). Using this plugin, the traces for each primary dendrite originated at the center of the cell soma. Fork points were then created along these primary dendrites and the secondary and tertiary arborizations were also traced. We performed the sholl analysis function within SNT using a 10um interval for concentric rings (Sholl, 1953).

Electrophysiology. Whole-cell patch-clamp recordings of miniature EPSCs (mEPSCs) were obtained from DIV14-16 dissociated mouse cortical neurons infected with either AAV-dsRed or AAV-mCherry-p2A-cre. Recordings were obtained at room temperature in a HEPES-buffered saline recording solution containing the following: 119mM NaCl, mM5 KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 30mM glucose, and 10mM HEPES, pH 7.2. The bath solution contained 1uM TTX (R&D Systems Inc. 1069), 10uM Bicuculline (R&D Systems, Inc. 0130), and 50uM D-APV (Tocris 0106). The electrode recording solution contained the following: 10mM CsCl, 105mM CsMeSO3, 0.5mM ATP, 0.3mM GTP, 10mM HEPES, 5mM glucose, 2mM $MgCl_2$, and 1mM EGTA, pH 7.2. Electrode resistances ranged from 2.5 to 4 MΩ and access resistances ranged from 5 to 20 MΩ. Cells were recorded at a holding potential of -70 mV with a calculated liquid junction potential of approximately 12.5mV. Signals were amplified, filtered to 2 or 5 kHz, and digitized at 10 kHz sampling frequency. mEPSCs were analyzed using ClampFit 10.3 (Molecular Devices) by averaging the amplitude and frequency of 150-300 events for each trace, with 5mV as the minimum threshold. Histograms were also generated for the collection of individual amplitudes and plotted as a cumulative probability.

Statistical analyses. Statistical differences between conditions were analyzed using either unpaired t tests (two groups) with indicated corrections for unequal variances or by ANOVA and indicated post hoc multiple-comparison test (>2 groups) conducted in Prism software (GraphPad).

D. Results

AAV1 and AAV2 serotypes reliably infect cultured neurons

After generating primary dissociated cultures from Nedd4-1 floxed mice as described in the Methods section (Figure 2.1 A), we added AAV virus to the cultures at DIV7 using a 1 :50 dilution of AAV1-Hsyn-dsRed (1.61E+11 titer units/mL) or a 1:10 dilution of AAV2-CAG-mCherry-p2A-Cre (2.76E+12 titer units/mL). Visualization of the cultures at DIV14 with a calcein cell health indicator demonstrated that the AAVs robustly infected axon-containing, dendritically branched cortical neurons and did not impact gross morphology (Figure 2.1 B).

Neurons infected with AAV2-mCherry-cre express cre recombinase protein

We verified that neurons infected with the AAV2-CAG-mCherry-p2A-cre virus (AAV-cre; KO) expressed cre in addition to the RFP marker by fixing after 7 days of viral incubation, and immunostaining using an anti-cre recombinase antibody. We found that neurons infected with AAV2-CAG-mCherry-p2A-cre positively express cre recombinase (Figure 2.2), whereas neurons infected with a control reporter, AAV1-Hsyn-dsRed (AAV-dsRed; WT), do not (data not shown). We found that 100% of RFP+ cells co-expressed cre, and that on average, there was roughly 1 additional RFP negative, cre expressing cell per field of view (RFP+, 6.02 ± 0.440; cre+, 6.88 ± 0.436; $n = 41$, Wilcoxon matched-pairs signed rank test, ****$p < 0.0001$, values represent mean ± SEM) (Figure 2.2). This suggests a high level of confidence that when evaluating the effects of Nedd4-1 loss in single cells, RFP+ neurons

will indeed lack Nedd4-1. This also emphasizes the need to incorporate a separate reporter conditions into the experimental design since RFP- cells have a small but non-zero likelihood of containing cre virus and representing a falsely negative phenotype. Overall, this yields a promising approach to studying Nedd4-1 function in mature neurons.

AAV-expressed cre recombinase knocks out Nedd4-1 in viable dissociated cultures

To ensure that AAV-mediated expression of cre recombinase leads to the ablation of Nedd4-1 protein in neurons, we evaluated Nedd4-1 expression at DIV14, 7 days after addition of AAV. We found that neurons infected with AAV-cre expressed significantly less Nedd4-1 protein than did the AAV-dsRed controls in heterogenous cortical mixed cultures (Fig 2.3) as well as in single cells (Fig. 2.4) using an anti-Nedd4-1 antibody. In mixed cultures, we observed on average a roughly 70% reduction in Nedd4-1 protein expression (N = 3; $^{**}p = 0.0081$). In a single-cell analysis of Nedd4-1 expression using fluorescent microscopy, we observed a significant decrease in the mean gray value, or signal intensity, of the fluorescence measurement between cre negative neurons (WT; 94.44 ± 6.23 pixel intensity, $n = 20$; mean ± SEM) and cre positive neurons (KO; 34.5 ± 3.77 pixel intensity, $n = 20$; mean ± SEM) ($^{****}p < 0.0001$).

Dendritogenesis is maintained in mature neurons following AAV-mediated cre expression

Since Nedd4-1 is critically important in the development of dendrites, or dendritogenesis, we wanted to ensure that by introducing cre recombinase, thereby knocking down Nedd4-1, at a later developmental date, that we can circumvent the early impairments of dendritogenesis that come with a global Nedd4-1 knockout. We therefore assessed the

arborization patterns of WT and KO cultures at 2 and 3 weeks of age (DIV14 and DIV21) to determine whether Nedd4-1 KO impacted dendrite development. The sholl profiles of WT and KO generally exhibited similar curves over the 10-60um distance (Figure 2.6 B, E). We evaluated the number of primary dendrites, defined as neurites projecting directly from the cell body (including both apical and basal dendrites), measured at the first 10um concentric ring, and found no significant difference between WT and KO at both DIV14 (WT 8.848 ± 0.397 intersections, $n = 33$; KO 8.424 ± 0.369 intersections, $n = 33$; $^{ns}p = 0.4367$) and DIV21 (WT 8.000 ± 0.457 intersections, $n = 31$; KO 7.125 ± 0.297 intersections, $n = 32$; $^{ns}p = 0.1112$) (Figure 2.6 C, F). Although there appears to be a trend in which KO neurons may have a slightly less pronounced distal arborization, defined as neurite intersection at a 60um distance from the cell soma, no significant difference could be detected within the error of our experiment at DIV14 (WT 29.818 ± 1.678 intersections, $n = 33$; KO 26.818 ± 1.403 intersections, $n = 33$; $^{ns}p = 0.1748$) (Figure 2.6 F). However, this effect does become more pronounced at DIV21, where we observe a significant difference in the distal arborization of WT and KO neurons (WT 28.258 ± 1.433 intersections, $n = 31$; KO 23.844 ± 1.331 intersections, $n = 32$; $^{*}p = 0.0274$) (Figure 2.6 G). This suggests that in addition to the novel functions Nedd4-1 begins to play in later stages of neuronal maturation, it could be also maintain its lasting role in neurite development, especially in the distal regions, well after the window of early development. Since here we can capture a period of time between DIV14 and DIV21 in which WT and KO look phenotypically similar, we perform all of our single-cell experiments at DIV14-16.

Nedd4-1 ablation does not impair spine morphology

Since neuronal dendrite growth is not significantly impaired in the Nedd4-1 KO at DIV14, we next assessed the density of spines on these KO dendrites. We co-expressed our neuronal cultures with either AAV1-dsRed or AAV2-mCherry-cre in combination with a sindbis 2gene-GFP virus that robustly fills spines. This allows us to reliably visualize RFP-expressing cells using a single marker to quantify spines, as the RFP fill from AAV1-dsRed AAV2-mCherry-cre does not fill the dendrites and spines to a reliable extent (data not shown). We found that KO neurons possess spine characteristics similar to that of WT (Figure 2.7) (Density: WT 0.960 ± 0.0378, KO 0.992 ± 0.0500 spines per micron, n = 31, 31; Height: WT 15.2 ± 0.266, KO 15.7 ± 0.296 um, n = 595, 615; Width: WT 5.10 ± 0.0615, KO 5.11 ± 0.0636 um, n = 595, 615). These characteristics were not statistically different, assessed by unpaired t-tests to the capture the difference between WT KO conditions, with Welch's correction for unequal variance when appropriate (Density $^{ns}p = 0.6084$; Height $^{ns}p = 0.2604$, Width $^{ns}p = 0.9270$).

Nedd4-1 ablation does not impair synaptic protein expression

The presence of spines in cortical neurons implies but does not necessitate the presence of synaptic or electrical activity, so we separately stained for two synaptic architectural proteins, bassoon, a pre-synaptic marker, and PSD95, a post-synaptic marker, to ensure that the expression of such synaptic proteins was not grossly reduced in KO cultures. We qualitatively evaluated whether the abundance of the pre- and post-synaptic proteins in the vicinity of RFP+ WT or KO cells were altered via immunostaining and fluorescent imaging. We observed the presence of both bassoon and PSD95 on and around the dendrites of RFP-expressing neurons, indicating that the formation of spines and synapses in these

dissociated cultures is likely maintained (Figure 2.8). Without a co-stain of the two markers we cannot say with certainty whether these synapses are definitively expressed on the spines of AAV-infected neurons, or whether they are paired or unpaired synapses, and so further characterization is required to quantify the degree of synaptic contacts between the pre- and post- synaptic markers. However, this data provides a first approximation for the presence of synaptic markers in KO neurons.

Nedd4-1 ablation does not impair synaptic activity

In addition to the qualitative fluorescent immunostaining, we employed electrophysiological recordings to determine whether synapses and synaptic receptors were present and active in KO neurons. To further corroborate the above evidence that these dendritic, spine-containing KO neurons express active synapses, we measured the amplitude and frequency of miniature excitatory synaptic currents (mEPSCs) in WT and KO neurons. We found no significant difference between WT and KO in the amplitude (Figure 2.9 B; WT 14.38 ± 0.786 mV, n =30; KO 14.07 ± 0.568 mV, n = 31; Unpaired t-test p = 0.7503.) or frequency (Figure 2.9 C; WT 2.252 ± 0.223 Hz, n =30; KO 1.959 ± 0.214 Hz, n = 31; Unpaired t test, p = 0.3469) of mEPSCs, suggesting that KO neurons display similar electrophysiological properties to WT neurons. Because there were no changes in either amplitude or frequency, this suggest there are no changes in postsynaptic spine number and receptors, or presynaptic signal generation. Further, because these KO neurons exhibit normative levels of synaptic activity, they can be employed for further investigations into the effect of Nedd4-1 knockout on synaptic plasticity paradigms.

E. Discussion

Since it has been previously shown that early cre-mediated conditional Nedd4-1 knockout is embryonically lethal *in vivo* and gives rise to dendritic growth deficits *in vitro*, we sought to establish a model of Nedd4-1 knockout this gives rise to viable neuronal cultures with which we could investigate receptor trafficking in mature neurons. We found that driving Nedd4-1 knockout after 7 days in primary dissociated neuronal cultures leads to the development of neurons with robust dendritic arborization and intact synapses and synaptic activity. This suggests that by delaying cre-mediated knockout of Nedd4-1, we can bypass the critical window in which Nedd4-1 regulates developmental processes, and therefore study the non-developmental functions of Nedd4-1 in mature neurons.

As seen in this work, Nedd4-1 KO neurons display typical dendritic arborization, spines, synapses, and synaptic activity, therefore this system represents an adequate model for Nedd4-1 KO that we can use for further investigations. Thus, these viable cultures offer the much-needed opportunity to gather data on the effects of Nedd4-1 knockout on endogenous protein trafficking.

However, Nedd4-1 knockout in our cultures was not wholly without effect. In comparing the development of neurites and dendritic branching, we observed that the distal arborization of Nedd4-1 KO neurons began to diverge phenotypically from WT neurons at older ages, and that even at younger ages, dendritic branching trended in the same direction. Therefore, a balance must be made between expressing cre for a sufficient length of time to ensure complete knockout of Nedd4-1, and investigating experimental questions before any

unwanted off-target, Nedd4-1 dependent, deficits occur. Here we have shown that 7 days of AAV-mediated cre expression is a sufficient length of time to deplete the neurons of endogenous Nedd4-1 without negatively impacting overall dendrite growth. Thus, in the future we would recommend that AAV-cre be added to cultures only after DIV7 for research on the non-developmental functions of Nedd4-1. This opens new avenues to test novel functions of Nedd4-1 in neurodegeneration and disease states, and to explore the regulation of proteins ubiquitinated by Nedd4-1 in a developmentally matured system.

The foundational investigations of Nedd4-1 knockout by Kawabe et al. demonstrated that early knockout of Nedd4-1 in autaptic cultures, while significantly impacting dendritogenesis, exhibited no significant difference in the amplitude of mEPSCs, which is reflected in our data as well, suggesting that the receptor expression at synapses is independent of dendrite morphology. They did report on a significant decrease in mEPSC frequency, which we did not detect within the error of our experiments but saw a trend in that direction.

One supposition for knocking out a key contributor to receptor internalization long term is that a prolonged absence of this endocytic regulator could lead to a buildup of receptor on the surface. Given that we see similar mEPSC amplitudes between WT and KO, it is likely that separate mechanisms control the long-term, homeostatic maintenance of postsynaptic glutamate receptor expression. Nedd4-1 is likely involved in activity-induced internalization, as we have previously reported, as well as in regulating distinct molecular pathways yet undiscovered. For instance is has been suggested to associate with proteins in the ESCRT (endosomal sorting complexes required for transport) pathway, with implications of Nedd4-1

involvement in HIV (Human Immunodeficiency Virus) and MLV (Murine Leukemia Virus) viral budding pathways (Bartusch & Prange, 2016; Blot et al., 2004; Sette et al., 2010).

One caveat to this model is that because there is a wide diversity of neurons type in a mixed cortical culture, it difficult to confirm the exact cell-type specificity of individual neurons for biochemical experiments. Experimental design should take this into account, using additional cell-type specific markers in immunostaining experiments where possible, or ensuring that a sufficient number of samples are collected in order to be able to detect any significant differences that exist between conditions. Nonetheless, this culturing framework provides promising groundwork for future experiments to elucidate the role of Nedd4-1 in many forms of plasticities and behaviors *in vitro*. Encouragingly, this method does lend itself well to use in organotypic slices and *in vivo*, where it will be easier to identify specific brain regions and cell-types. It will be very rewarding to see AAV-cre used in future works employing acute brain slices to study network dynamics in intact circuits and *in vivo* to study Nedd4-1 dependent behaviors.

F. Acknowledgements

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Chapter 2, in full is currently being prepared for submission for publication of the material. Hoffner, Nicole; Ibarra, Lauren; Dozier, Lara; Patrick Gentry N. The thesis author was the primary investigator and author of this material.

G. Figures

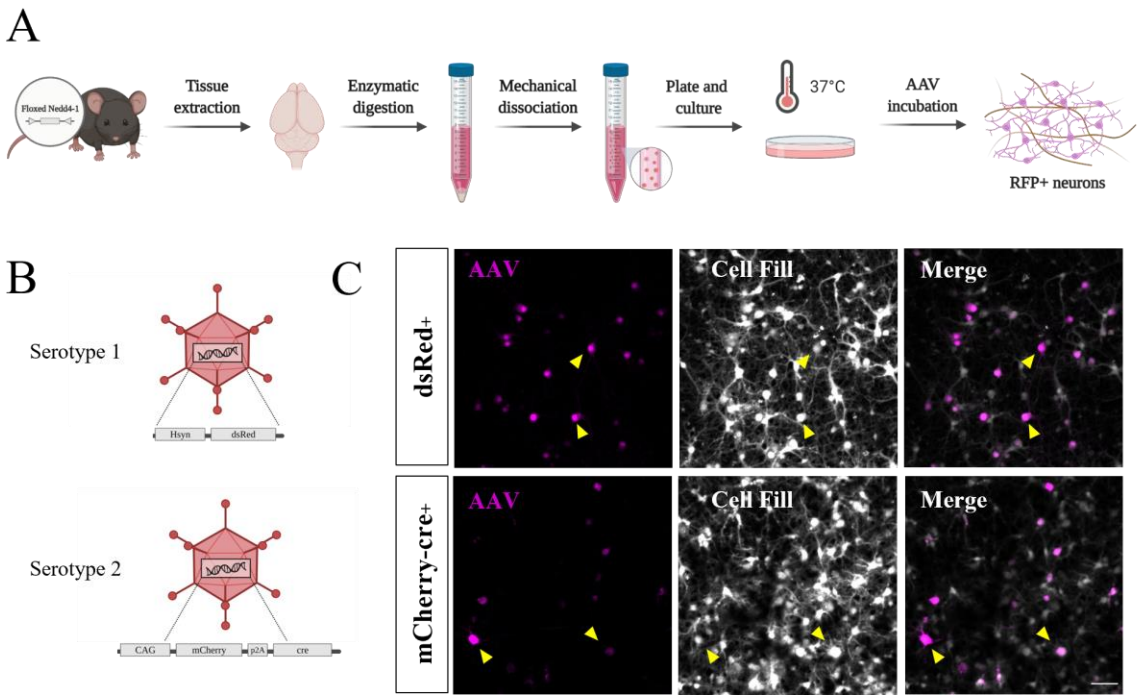

Figure 2.1. Generation of dissociated primary cortical neurons and expression of AAV constructs. (**A**) Schematic of primary dissociated culture preparation using cortical and hippocampal tissues extracted from homozygous flox Nedd4-1-expressing animals. Tissues were extracted, digested, dissociated, and plated for 7-10 days before addition of AAV virus. Neurons were transduced with AAV-delivered vectors expressing Hsyn-dsRed (**B, top**) or CAG-mCherry-p2A-cre (**B, bottom**). (**C**) DIV14 neurons incubated 7-10 days with AAV leads to robust infection of neurons and expression of appropriate RFPs. Yellow triangles indicate infected neurons within a dissociated culture filled with fluorescent cell fill. Scale bar 50um. *Schematics made with Biorender*.

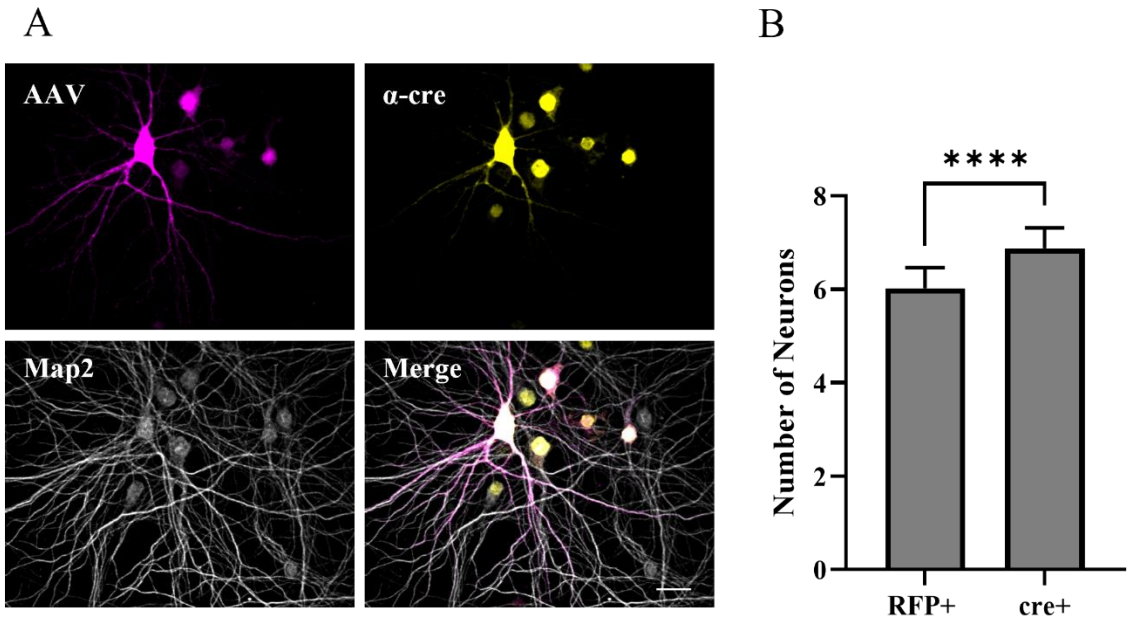

Figure 2.2. AAV2-mCherry-p2A-cre infected cells express cre. (**A**) Representative image indicating that AAV-mCherry-cre infected neurons are Cre+ via anti-cre recombinase immunostaining and verification of neuron status using anti-Map2. (**B**) Quantification of the average number of RFP+ cells and cre+ cells in a single field of view. 100% of RFP+ cells expressed the cre recombinase protein (****$p < 0.0001$). Scale bar 10um.

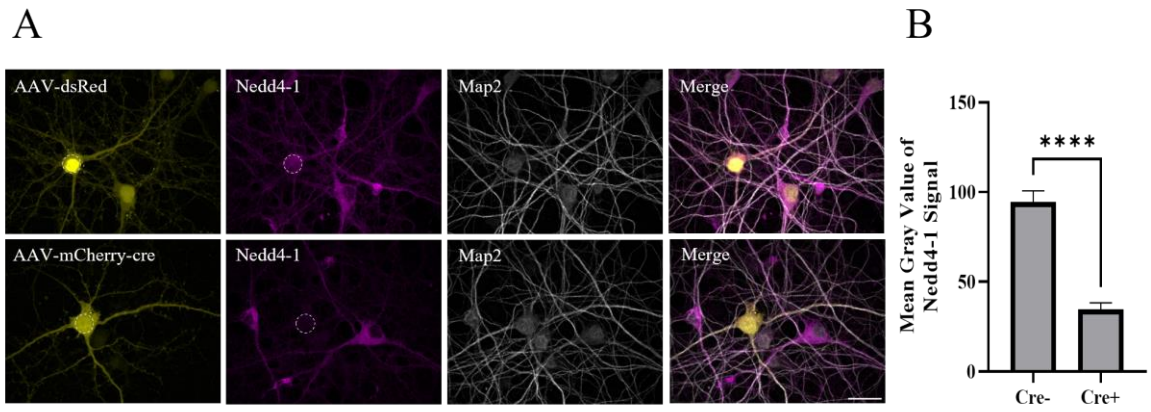

Figure 2.3. AAV-mediated knockout of Nedd4-1 in single cells. (**A**) Representative images of AAV-infected cells from control (top) and Nedd4-1 KO (bottom) immunostained for Nedd4-1 and Map2. White circles indicate representative areas used for quantification. (**B**) Quantification of Nedd4-1 protein expression levels, as intensity of α-Nedd4-1 immunostaining, in control (94.4 ± 6.23 pixel intensity) and AAV-cre infected cells (34.5 ± 3.77 pixel intensity) Bars represent mean values; error bars represent SEM, ****$p < 0.0001$.

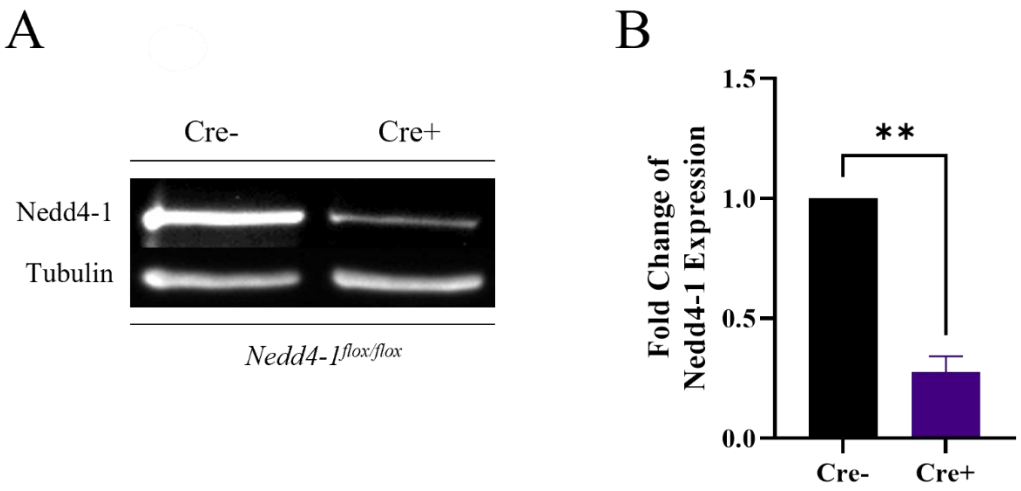

Figure 2.4. AAV-mediated knockout of Nedd4-1 in mixed cultures. (**A**) Total protein lysates were collected from control or AAV-cre infected dissociated, and we observe that total Nedd4-1 protein levels are reduced in cre+ cultures. (**B**) Quantification of Nedd4-1 expression levels in AAV-dsRed infected vs AAV-mCherry-cre infected cells, normalized to control. Bars represent mean values; error bars represent SEM, $^{**}p < 0.01$, N = 3 independent culture preparations.

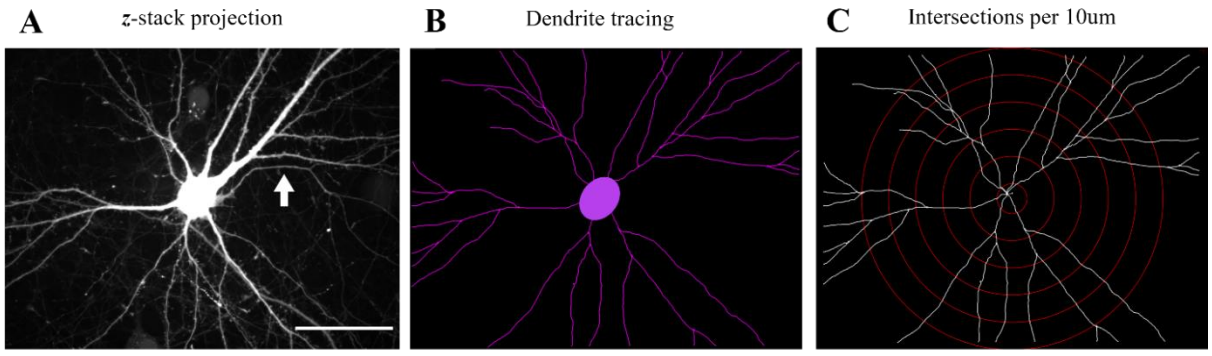

Figure 2.5. Sholl analysis method to quantify dendritogenesis. (**A**) Projected *z*-stack image. (**B**) 8-bit trace of dendrites, constructed using the Fiji plugin Simple Neurite Tracer (SNT). Axons, an example indicated by the white arrow in *A*, were excluded in tracings, and the cell soma is represented here for visualization only. (**C**) Projected *z*-stack image overlaid with an example of concentric rings, spaced 10 **µ**m apart, centered on the cell soma. This image can be used for manual counting of dendritic crossings (intersections) or used in SNT for automated counting of dendritic crossings. Scale bar: 50 **µ**m.

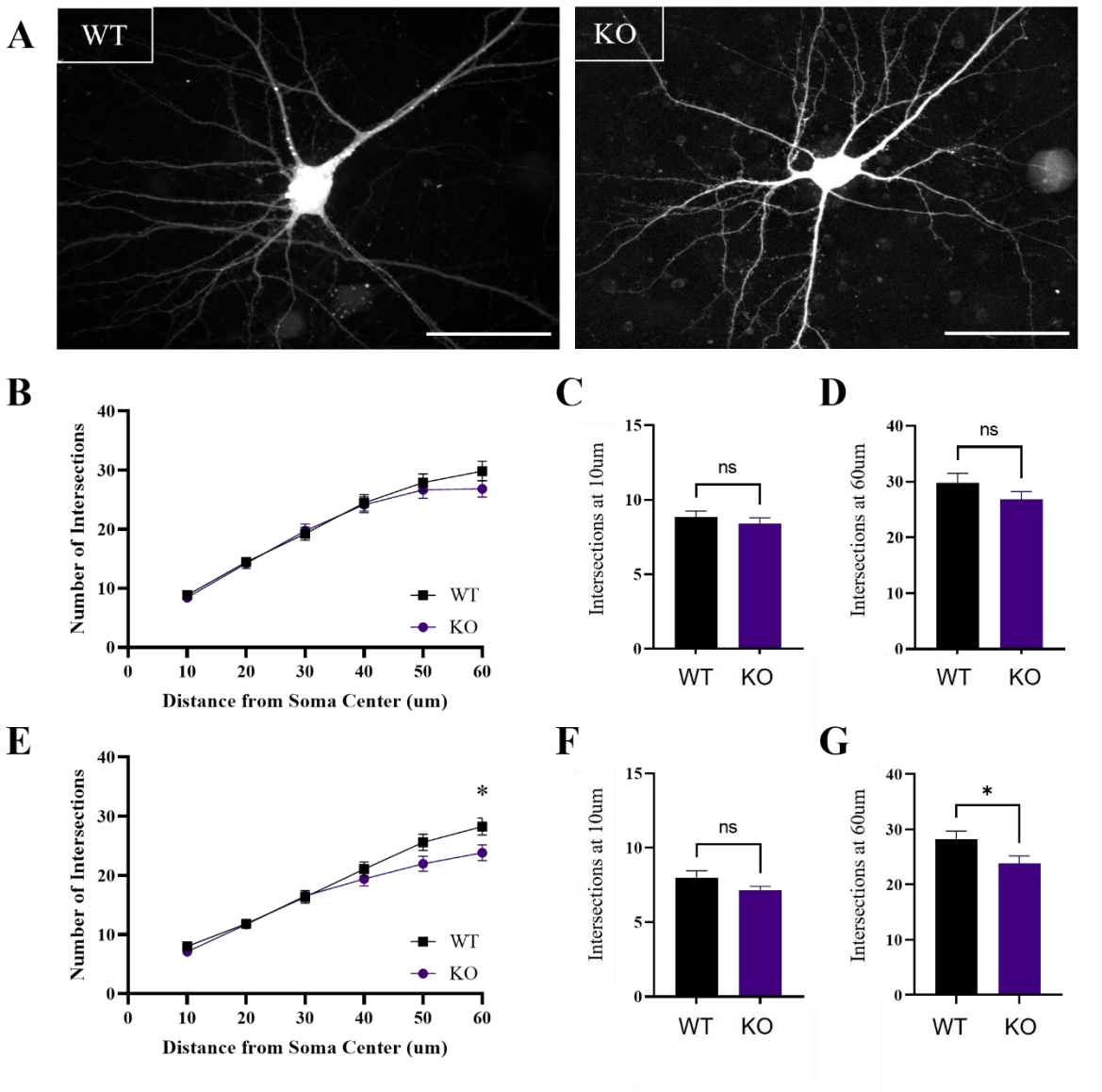

Figure 2.6. Nedd4-1 ablation impairs distal dendritogenesis in older neurons under longer periods of knockout. A comparison of the effect of Nedd4-1 ablation on neurite development extending to older aged neurons. Neurons were infected with AAV at DIV7 and aged to either DIV14 or DIV21. (**A**) Representative images from DIV14 WT and KO cultures. (**B**) Sholl profile of WT and KO branching at DIV14, with (**C**) quantification of primary neurites (WT 8.848 ± 0.397 intersections, $n = 33$; KO 8.424 ± 0.369 intersections, $n = 33$; $p = 0.4367$) and (**D**) distal arborization (WT 29.818 ± 1.678 intersections, $n = 33$; KO 26.818 ± 1.403 intersections, $n = 33$; $p = 0.1748$) highlighted. Similarly, (**E**) Sholl profile of WT and KO branching at DIV21, with (**F**) quantification of primary neurites (WT 8.000 ± 0.457 intersections, $n = 31$; KO 7.125 ± 0.297 intersections, $n = 32$; $p = 0.1112$) and (**G**) distal arborization (WT 28.258 ± 1.433 intersections, $n = 31$; KO 23.844 ± 1.331 intersections, $n = 32$; $*p = 0.0274$) highlighted. Scale bar 50um.

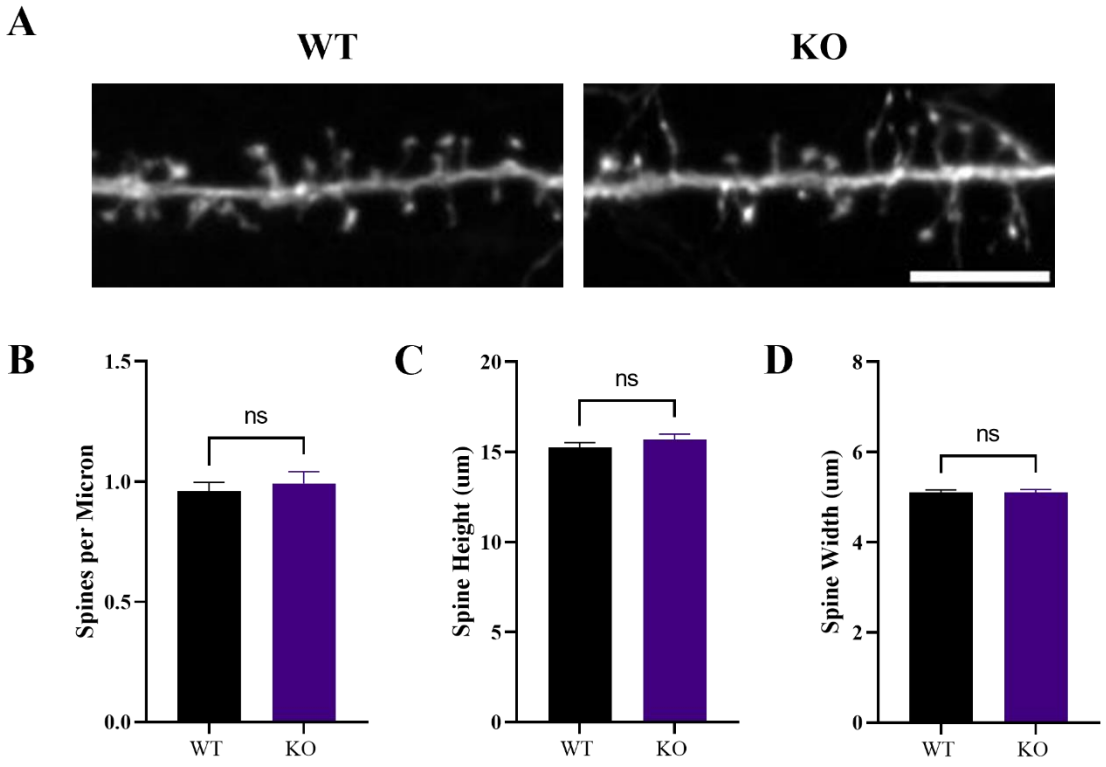

Figure 2.7. Nedd4-1 KO neurons have spine characteristics similar to that of WT neurons. (**A**) Representative images from AAV-infected cells from dsRed WT control (left) and mCherry-cre Nedd4-1 KO (right) showing the dendrite segments of DIV14, IV7 neurons used for spine counting using an additional GFP fill. (**B,C,D**) Quantification of spine density, height, and width, respectively, shows that there is no significant difference between WT and KO (Spine Density: WT 0.960 ± 0.0378 spines per micron, KO 0.992 ± 0.0500 spines, $^{ns}p > 0.05$, $n = 31$ for each condition; Spine Height: WT 15.2 ± 0.266 um, KO 15.7 ± 0.296 um, $^{ns}p > 0.05$, $n = 595, 615$, respectively; Spine Width: WT 5.10 ± 0.0615 um, KO 5.11 ± 0.0636 um, $^{ns}p > 0.05$, $n = 595, 615$, respectively). Scale bar 5um.

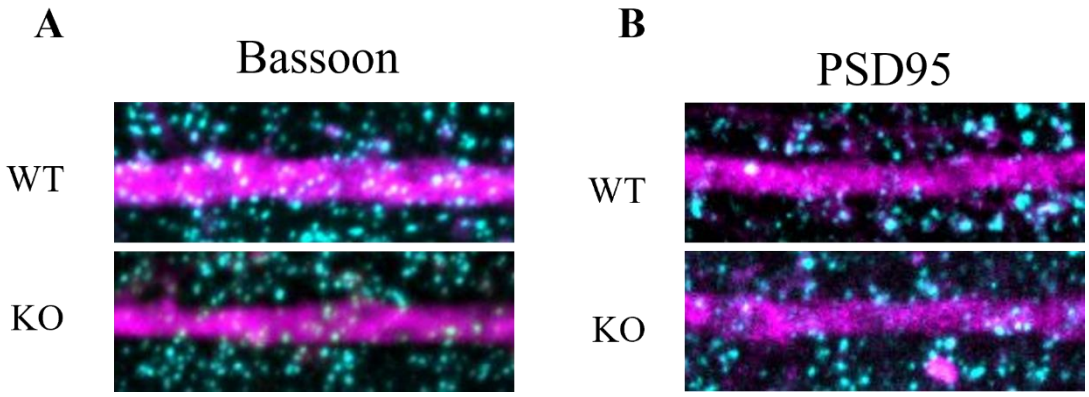

Figure 2.8. Nedd4-1 KO neurons express the pre- and post-synaptic markers Bassoon and PSD95. Immunostained dendrites from DIV14, IV7 cortical neurons infected with either AAV-dsRed (WT) or AAV-mCherry-cre (KO). (**A**) Bassoon signal (cyan) is in close proximity to spines in WT and KO neurons (yellow arrows). (**B**) Similarly, the post-synaptic marker PSD95 (cyan) is expressed on the spines of both WT and KO neurons.

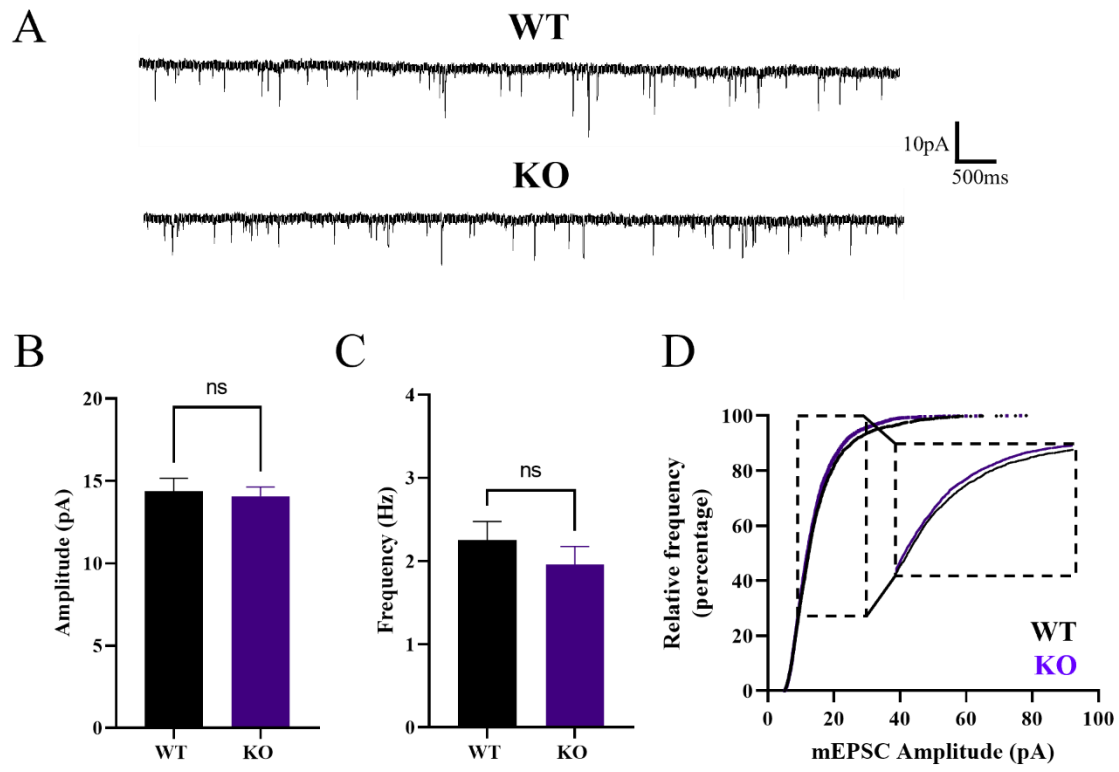

Figure 2.9. Nedd4-1 KO neurons exhibit normal synaptic activity. (**A**) Representative traces of mEPSCs recorded from dissociated cortical and hippocampal neurons. (**B)** Quantification of mEPSC amplitude in WT and KO neurons, demonstrating that mEPSC amplitude and frequency of KO neurons is not significantly different than WT; graphs show mean ± SEM, Unpaired t-test, p = 0.7503, n = 30 & 31 cells over 7 independent cultures. Scale bar, 10 pA and 500ms. (**C**) Similarly, frequency was not significantly different between WT and KO. Unpaired t-test, p = 0.3469. (**D**) Cumulative probability distributions of all mEPSC amplitudes recorded from WT and KO neurons. n = 3161 and 3855 events, respectively.

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Chapter III

Potent Small Molecule Inhibitor of Nedd4-1, 1-benzyl-indole-3-carbinol, in Neurons

A. Abstract

Protein ubiquitination, catalyzed by the E3 ligase Nedd4-1, is an important modulator of protein localization and function and therefore an underlying contributor to cellular health. The Nedd4-1 ubiquitin ligase carries out many critical functions underlying cell health and maintenance. In neurons, Nedd4-1 contributes to a number of diverse proteostatic processes, spanning from early development to late-stage degeneration. Short-term pharmacological inhibition of Nedd4-1 activity would provide a valuable method to probe the fast-timescale, activity dependent functions of Nedd4-1. Therefore we obtained a naturally occurring indole-3-carbanol (I3C) compound, known to have minimal impacts to cell health in oncology research, and found that neurons tolerate acute Nedd4-1 inhibition via I3C incubation. The synthetic analogue 1-benzyl-I3C (1BI3C) is a more potent variant with a significantly lower IC50 inhibitory concentration (I3C IC50 284uM, 1BI3C IC50 12.5uM) and found that neurons tolerate this variant as well. We found that 1BI3C indeed inhibits Nedd4-1 ubiquitin ligase activity with no ill effects on spine expression or synapse activity over a 24 hour incubation period. Our results indicate that 1BI3C is a promising tool for use in further investigations into Nedd4-1 function in neurons, using acute inhibition on shorter timescales.

B. Introduction

The ubiquitin proteasome system (UPS) is a mechanism that cells engage to carry out maintenance of protein quantity and quality. Damaged, non-functional, misfolded, and unneeded proteins must be processed correctly in order for cells to maintain health and

function. One process that contributes to the quality control of proteins in the cell involves the modification of proteins by tagging it with a ubiquitin molecule. This ubiquitin modification targets them to the UPS system, which processes the protein for its downstream degradative or recycling fate. Ubiquitination is carried out by a stepwise cascade of successive protein interactions, whereby an E1 ubiquitin activating enzyme binds to a ubiquitin molecule and transfers it to an E2 ubiquitin conjugating enzyme. Next the E2 ubiquitin conjugating enzyme transfers the ubiquitin molecule to an E3 ubiquitin ligase enzyme. This E3 ligase enzyme attaches, or ligates, the ubiquitin molecule onto the target protein, ie substrate. While in most eukaryotes there are relatively few E1 and E2 enzymes, there can be hundreds of E3 enzymes. This suggests that the E3 enzymes are responsible for conferring specificity in substrate ubiquitination and makes them attractive therapeutic targets. Once the appropriate number and confirmation of ubiquitin molecules are attached to the substrate, downstream functional consequences occur: depending on the type of conformation and attachment, this protein ubiquitination signals for protein endocytosis, translocation to intracellular transport vesicles, leading to protein degradation or recycling back to the membrane, and lastly, could also contribute to the activation or availability of the function that the tagged protein can carry out.

Further, protein ubiquitination takes many forms. These include mono-ubiquitination, multimono-ubiquitination, homotypic polyubiquitination, and heterotypic polyubiquitination of a substrate, meaning it can be bound with one ubiquitin molecule, many single ubiquitin molecules, or a ubiquitin molecule that is itself ubiquitinated, forming chains. These heterotopic polyubiquitin chains take exist in mixed chains, branched chains, the inclusion of ubiquitin-like modifiers such as SUMO and NEDD8 attachments, as well as chemical

modifications to the ubiquitin chains, like phosphorylation and acetylation. Mono-ubiquitination is thought to lead to processes including endocytosis, proteins sorting, compartmental trafficking, and DNA repair. Polyubiquitin branches contain ubiquitin molecular attached to other ubiquitin molecules at specific lysine amino acid sites, and both the shape of this branching as well as the site location of the attachment dictates the functional consequences. For example, ubiquitin at lysine 48 (K48) leads to protein degradation, whereas ubiquitin binding at lysine 63 (K62) leads to DNA repair, endocytosis, and NF-κB activation. Through these combinatorial effects, ubiquitin plays an important role in protein regulation and health. In the brain, protein ubiquitination contributes to processes including proteostasis, inflammation, mitophagy, DNA damage repair, and receptor trafficking.

Neural precursor cell-expressed, developmentally down-regulated protein 4-1 (Nedd4-1) is one such enzyme that regulates receptor trafficking, therefore directly contributing to neuronal activity. It is critical for neuronal communication to have an appropriate balance of receptors at the surface. Processes underlying this availability include learning-dependent and activity-dependent synaptic changes as well as long-term homeostatic maintenance. Nedd4-1 contributes to this homeostatic maintenance by responding in an activity dependent manner to tag AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) for endocytosis. AMPARs are one of the main driving forces behind the communication between neurons, and so Nedd4-1 in general contributes to the processes underlying the availability of neurons to communicate.

Because Nedd4-1 is such an integral enzyme for cell health and maintenance at all stages of development, employing genetic means to abolish its protein expression has proven

difficult. Global knockout of Nedd4-1 is embryonically lethal, and even partial knockout can give rise to gait abnormalities and motor deficits.

In order to carry out its function, Nedd4-1 contains three important functional domains (Figure 3.1). It contains a C2 phospholipid binding domain, which helps target it to phospholipid membranes, putting it in proximity to membrane receptors. Second, it has four of WW (tryptophan-tryptophan) substrate binding domains, conferring specificity in which targets it modifies. Lastly it has a HECT (Homologous to the E6-AP Carboxyl Terminus) catalytic domain, which catalyzes the transfer reaction of the ubiquitin molecule bound to Nedd4-1 to the substrate protein to be modified. This catalytic HECT domain is the ideal targeting domain for inhibition of Nedd4-1 activity.

Indole-3-carbinol (I3C) is a compound that binds to Nedd4-1's HECT domain (Figure 3.1) to inhibit its catalytic activity (Nguyen et al., 2010). This pharmacological inhibition provides us with a tool to investigate Nedd4-1 function acutely, especially in dissociated neuronal cultures where drug application is easily accessible to neurons, which are rapidly responsive to such treatments. In addition to the naturally occurring I3c, there are synthetic derivatives with higher potency, as measured by a lower IC50, or concentration of drug required to reach 50% inhibition of the target (Quirit et al., 2017). Using these more potent variants of I3C, such as the synthetic derivative 1-benzyl-I3C (1BI3C), would provide a complementary tool to genetic ablation studies. However, it has not previously been shown whether neurons tolerate I3C or I3C synthetic derivatives, and so we show here that 1BI3C inhibits Nedd4-1 activity with minimal impact to the health and morphology of neuronal

cultures. Therefore, we plan to use 1BI3C in future work to inhibit Nedd4-1 activity and probe for its function in synaptic plasticity.

Interestingly, I3C is found in many common vegetables such as broccoli, brussels sprouts, and cauliflower, and much attention has been paid to the potential for I3C as a preventive intervention for certain types of cancers. Further research into Nedd4-1 function, using I3C, will be contribute to future work in understanding its potential for therapeutic treatments.

C. Materials and Methods

Dissociated Hippocampal Cultures: Neuronal cultures were generated from dissociated rat hippocampal or cortical neurons obtained from postnatal day 1 pups of either sex. Dissociated neuron suspension solution was plated onto poly-D-lysine-coated coverslips for immunostaining and confocal imaging, or poly-D-lysine-coated 6-well plastic dishes for biochemical Western Blotting experiments. Cultures were maintained in B27 supplemented neurobasal medium (Invitrogen) until use at 14-21 days in vitro (DIV), as described previously (Djakovic et al., 2009).

In vitro ubiquitination assay. Nedd4-1 in vitro self-ubiquitylation assay was carried out as previously described (Rossi et al., 2005). Briefly, the ubiquitylation reaction mixture contained 25 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 2.5 mM ATP, 4 mM MgCl2, 2 µl of bacterial purified wheat E1, 0.1 µg of a human UbcH7, 100 ng of bacterially purified recombinant Rat wild-type Nedd4-1 or catalytic inactive Nedd4-1 (CS) mutant, and 5

μg of Flag-tagged ubiquitin. After incubation for 90 min at 30 °C, the reactions were terminated by adding SDS loading buffer, resolved by SDS-PAGE, followed by immunoblot with anti-ubiquitin antibodies.

Western blot analysis. Total protein lysates were generated by scraping cells into RIPA buffer (50 mM Tric-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors and incubated for 20 minutes, rotating at 4°C. Protein concentration was determined by BCA protein assay (Fisher Scientific 23225) and was normalize prior to sample buffer addition. Samples were boiled with sample buffer, resolved on 4-12%, Tris-Glycine gel (Invitrogen Novex Mini Protein Gel XP04122), transferred to nitrocellulose paper, and probed with primary antibodies. Blots were digitized and band intensities were quantified using Fiji (ImageJ). Band intensities were normalized to the band mean intensity of tubulin from each respective sample.

Immunocytochemistry: DIV14-21 neurons were washed with cold PBS-MC (phosphate buffered saline + magnesium and calcium) 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. Tables 1 and 2 list the primary and secondary antibodies used for immunocytochemistry, respectively. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 1 hour at room temperature. Coverslips were mounted onto glass slides for confocal imaging.

Primary and secondary antibodies. Map2 Ck 1:20000 Abcam Inc. ab5392; Ms-488 Goat 1:1000 Life Technologies A11001; Rb-488 Goat 1:1000, Life Technologies A11034; Rt-488 Goat 1:1000 Fisher Scientific A11006; Ck-647 Goat 1:1000 Fisher Scientific A21449.

Confocal microscopy. All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nikon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 m/pixel at 1X), Plan Apochromat 40X/1.0 or 63X/1.0 numerical aperture objective, and an argon/krypton 100mW air-cooled laser for 488/568/647nm excitations. Maximum projected confocal Z-stacks were analyzed with ImageJ. Statistical significance was determined through unpaired t tests or ANOVA with specified post hoc multiple-comparisons test using Prism software (GraphPad).

Electrophysiology. Whole-cell patch-clamp recordings of miniature EPSCs (mEPSCs) were obtained from DIV14-16 dissociated mouse cortical neurons infected with either AAV-dsRed or AAV-mCherry-p2A-cre. Recordings were obtained at room temperature in a HEPES-buffered saline recording solution containing the following: 119mM NaCl, mM5 KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 30mM glucose, and 10mM HEPES, pH 7.2. The bath solution contained 1uM TTX (R&D Systems Inc. 1069), 10uM Bicuculline (R&D Systems, Inc. 0130), and 50uM D-APV (Tocris 0106). The electrode recording solution contained the following: 10mM CsCl, 105mM CsMeSO3, 0.5mM ATP, 0.3mM GTP, 10mM HEPES, 5mM glucose, 2mM $MgCl_2$, and 1mM EGTA, pH 7.2. Electrode resistances ranged from 2.5 to 4 MΩ and access resistances ranged from 5 to 20 MΩ. Cells were recorded at a holding potential of -70 mV with a calculated liquid junction potential of approximately 12.5mV. Signals were

amplified, filtered to 2 or 5 kHz, and digitized at 10 kHz sampling frequency. mEPSCs were analyzed using ClampFit 10.3 (Molecular Devices) by averaging the amplitude and frequency of 150-300 events for each trace, with 5mV as the minimum threshold. Histograms were also generated for the collection of individual amplitudes and plotted as a cumulative probability.

Statistical analyses. Statistical differences between conditions were analyzed using either unpaired t tests (two groups) with indicated corrections for unequal variances or by ANOVA and indicated post hoc multiple-comparison test (>2 groups) conducted in Prism software (GraphPad).

D. Results

I3C does not disrupt cell health

First, we wanted to determine whether our dissociated neuronal cultures would tolerate a 24-hour treatment with I3C (Figure 3.2). DIV21 rat hippocampal neurons were treated with a range of concentrations of I3C, with the solvent DMSO as a control, or 40uM 1BI3C for 24 hours. Neurons were also infected with Sindbis GFP as a fluorescent fill for visualization. We found that qualitatively, the neuronal dendritic arborization morphology was maintained under these conditions. Because the higher concentrations of I3C did not appear to kill the neurons, we assessed neuron health against the more potent analogue 1BI3C (Figure 3.2). DIV21 rat hippocampal neurons were treated with 40uM 1BI3C or a DMSO control for 24 hours. Neurons were also infected for with Sindbis GFP as a fluorescent fill for

visualization. Similarly, we found that treatment with 1BI3C did not negatively impact neuronal cell health when treated with 1BI3C either.

1BI3C causes inhibition of Nedd4-1 auto-ubiquitination

Using the *in vitro* auto-ubiquitination assay of Nedd4-1 ubiquitin ligase purified by affinity immunoprecipitation allows us to determine whether *in vitro* ubiquitination of Nedd4-1 is blocked by the 1BI3C inhibitor. We found that Nedd4-1 auto-ubiquitination is robustly inhibited by 40uM of 1BI3C (Figure 3.4).

1BI3C does not alter spine morphology

To test whether a 24 hour incubation of 1BI3C alters neuron spine morphology, we treated DIV21 rat hippocampal neurons with 40μM 1-benzyl-I3C or DMSO control for 24 hours (Figure 3.5). Neurons were infected with Sindbis GFP as a fluorescent fill for visualization. After fixing, staining, and confocal imaging, neuronal dendrites were straightened, cropped to 35um segments, and spine morphology was assessed. We found no significant difference between spine density (DMSO 3.58 ± 0.105 spines, 1BI3C 3.67 ± 0.129 spines, Unpaired t-test $^{ns}p = 0.5714$, $n = 20$ dendrite segments), spine height (DMSO 13.3 ± 0.196 um, 1BI3C 13.4 ± 0.199 um, Unpaired t-test $^{ns}p = 0.6005$, $n = 749, 772$ spines, respectively), or spine width (DMSO 5.04 ± 0.0615 um, 1BI3C 5.01 ± 0.163 um, Unpaired t-test $^{ns}p = 0.7220$, $n = 749, 772$ spines, respectively) between conditions that.

1BI3C does not alter synaptic activity

Lastly, we recorded synaptic activity from neurons that were treated with DMSO or 1BI3C for 24 hours to determine if drug treatment led to any changes in neuronal activity (Figure 3.6). We found no significant difference between the two conditions with respect to either mEPSC amplitude (DMSO 12.1 ± 1.30 pA, 1BI3C 12.8 ± 1.27 pA, Unpaired t-test ^{ns}p = 0.7087, n = 6, 7 neurons, respectively) or frequency (DMSO 2.10 ± 0.900 Hz, 1BI3C 2.03 ± 0.614 Hz, Unpaired t-test ^{ns}p = 0.9487, n = 6, 7 neurons, respectively).

E. Discussion

Inhibition of Nedd4-1, a HECT domain-containing E3 ubiquitin ligase, via the use of small molecule inhibitors, is an emerging strategy to interrogate its potential as a therapeutic compound in cancer studies. Because Nedd4-1 plays such an important role in synaptic plasticity as well, it is essential to have the tools we need to study its function in neurons. We were interested in using the highly potent synthetic derivative of I3C, 1BI3C in neurons to further evaluate Nedd4-1's role in synaptic plasticity. As such, we aimed to characterize its suitability to use in our *in vitro* experiments in dissociated neuronal cultures. We examined the *in vitro* inhibition of enzymatic activity of purified Nedd4-1 by 1BI3C and found that 40uM 1BI3C robustly blocks the auto-ubiquitination of Nedd4-1, assessed by western blotting. Before use in neurons, we wanted to evaluate whether 1BI3C impairs neuronal growth or activity, and encouragingly, our immunocytochemistry data demonstrates that 1BI3C does not significantly alter the overall morphology of neuronal dendrites, spines, or synaptic activity. This highly potent variant of I3C, 1BI3C, promises to be a viable option for inhibiting Nedd4-1 ubiquitin ligase activity in neurons for future experiments.

F. Acknowledgments

Thank you to the entire research group – Dr. Gentry Patrick, Lara Dozier, Dr. Steve Gilmore, and Kevin White, for their valuable input and contributions to the work in this chapter.

Chapter 3 includes coauthored material. Hoffner, Nicole; Gilmore, Steven; Dozier, Lara; Patrick, Gentry. Chapter 3, Potent Small Molecule Inhibitor of Nedd4-1, 1-benzyl-

indole-3-carbinol, in Neurons. The thesis author was the primary investigator and author of this material.

G. Figures and Tables

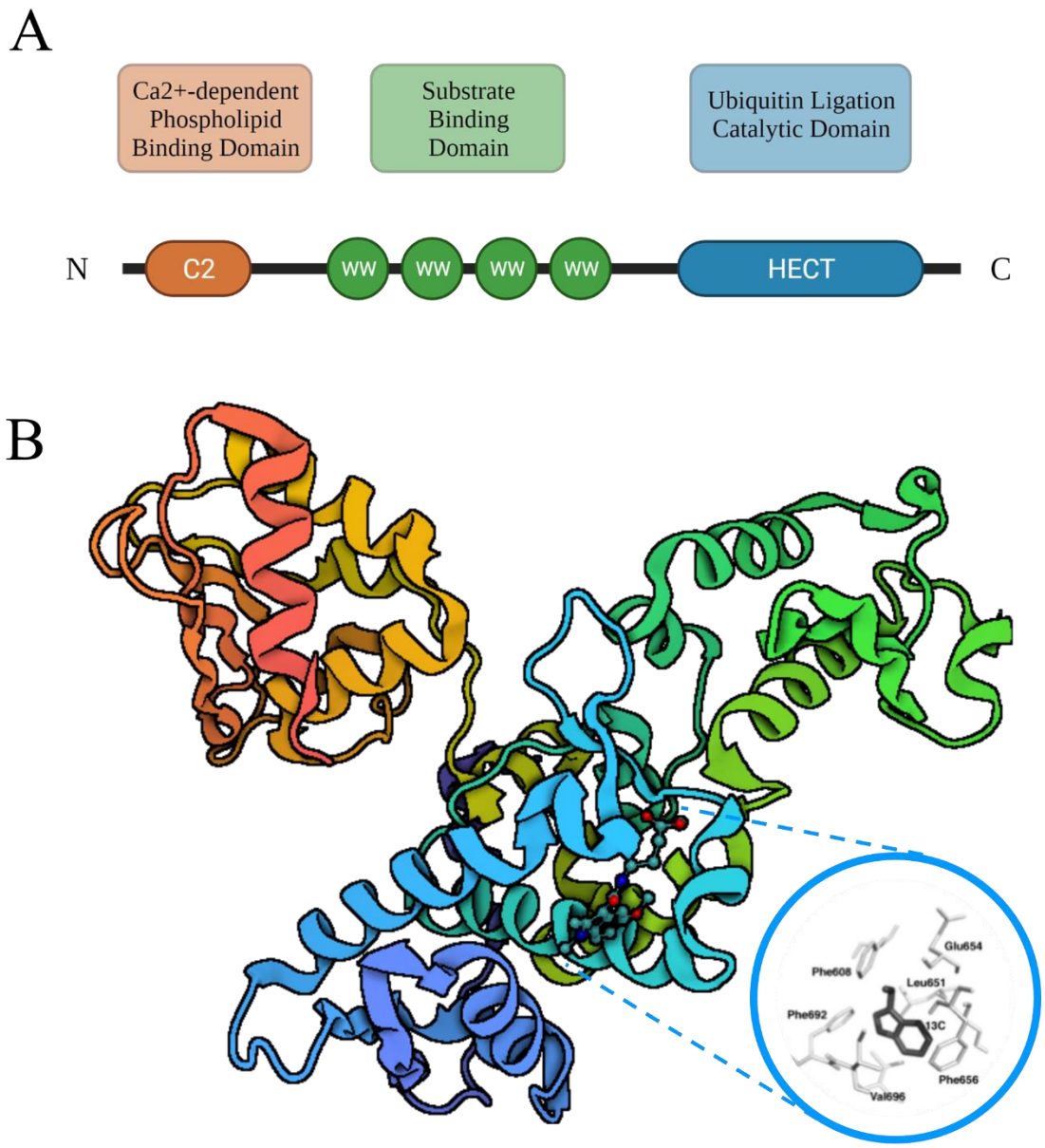

Figure 3.1. Representation of Nedd4-1 protein structure and indole-3-carbinol based inhibition of Nedd4-1 HECT catalytic domain. (**A**) Structure of Nedd4-1, highlighting the calcium-binding C2 domain, 4 WW domains that confer substrate specificity, and the catalytic HECT domain (**B**) Naturally occurring I3C, and its 1-benzyl-I3C analogue, is a small molecule inhibitor of Nedd4-1 ubiquitin ligase activity that inhibits the catalytic HECT domain of Nedd4-1 and therefore impede ubiquitin ligase activity. *Made with Biorender*.

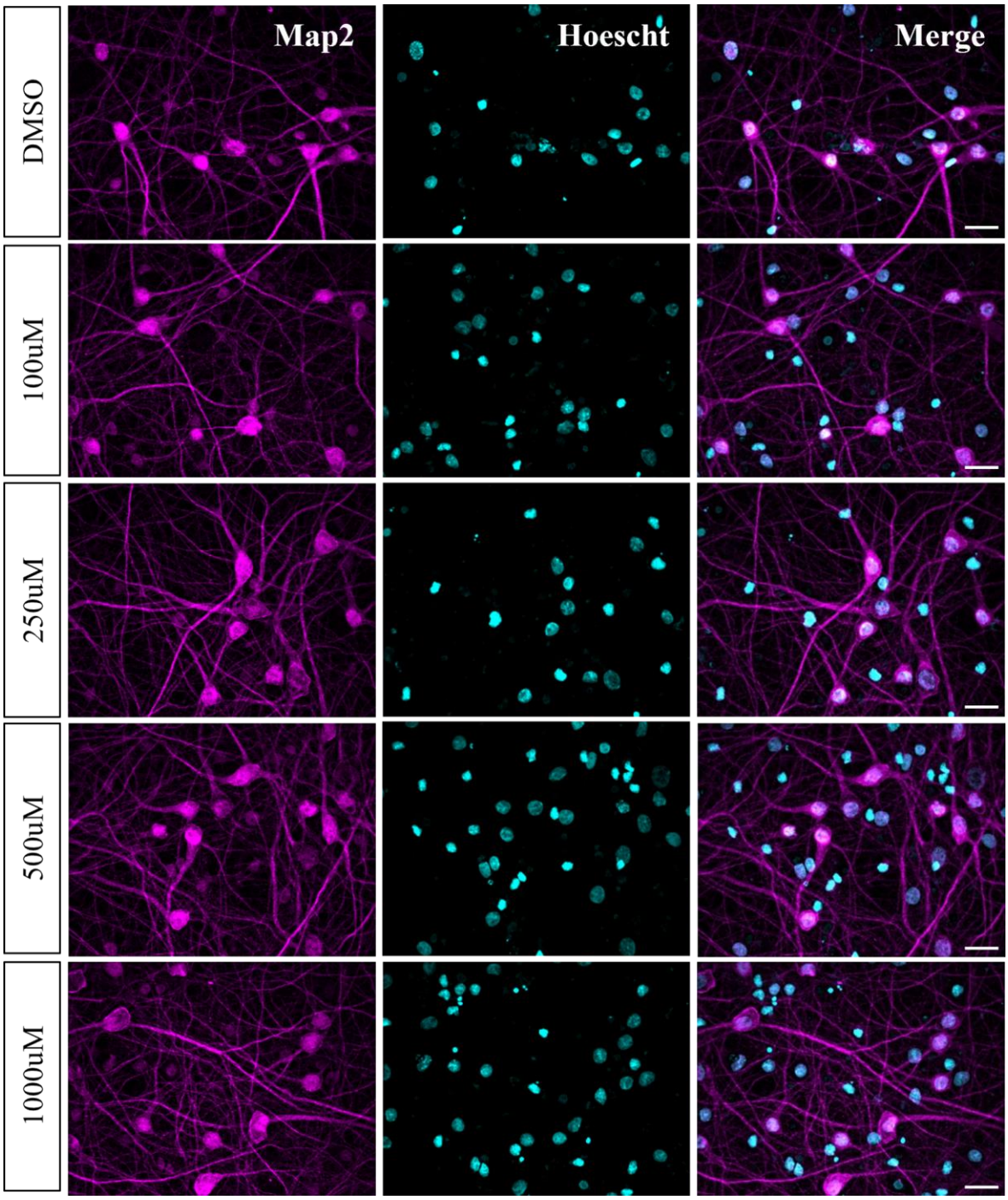

Figure 3.2. Cell health is not compromised after 1 hour incubation with the natural analogue of indole-3-carbinol. MAP2 immunostaining and Hoechst nuclear staining of dissociated hippocampal neurons after treating with the indicated concentration of I3C for 1 hour at the indicated concentrations. Scale bar 20um.

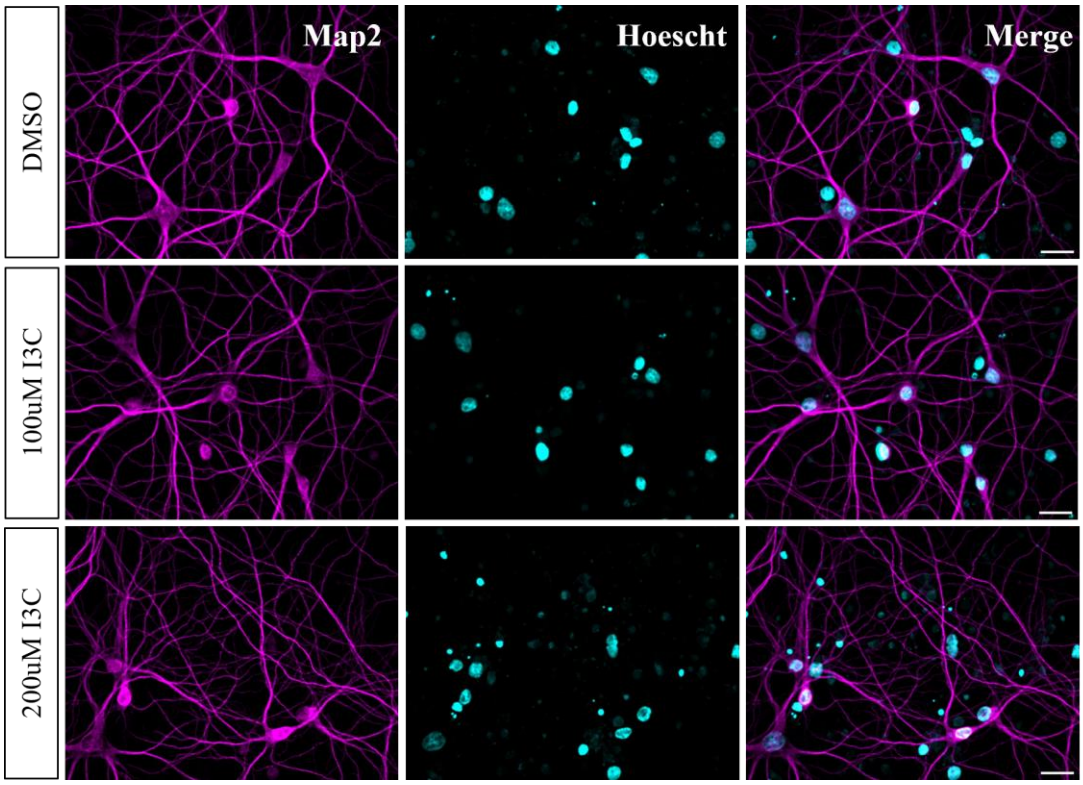

Figure 3.3. Cell health is not compromised after 24 hour incubation with the natural analogue of indole-3-carbinol. MAP2 immunostaining and Hoechst nuclear staining of dissociated hippocampal neurons after treating with the indicated concentration of I3C for 24 hours at the indicated concentrations. Scale bar 20um.

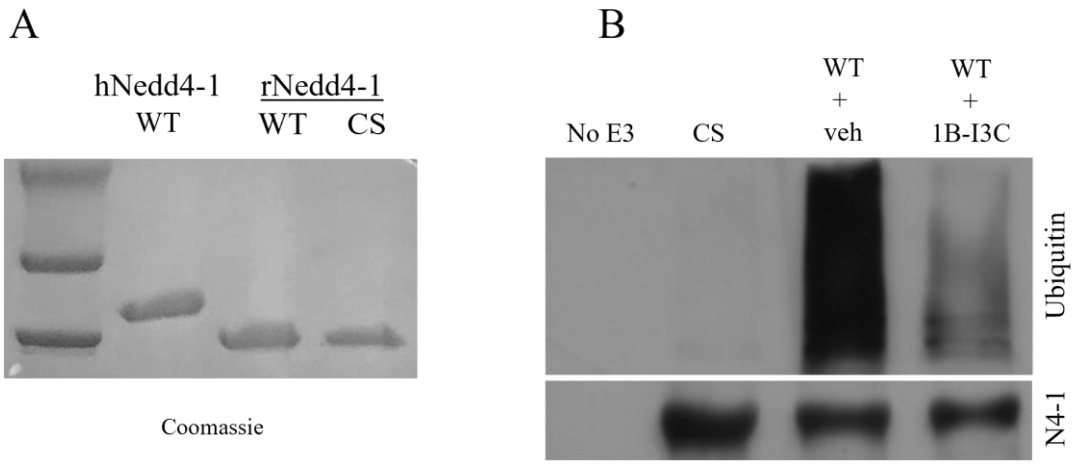

Figure 3.4. 1-benzyl-indole-3-carbinol blocks Nedd4-1 autoubiquitination. (**A**) purified GST proteins were examined by Coomassie Blue staining. (**B**) 100 ng of bacterially purified recombinant Rat wild-type Nedd4-1 or catalytic inactive Nedd4-1 (CS) mutant was incubated with bacterial purified wheat E1 and a human UbcH7 for 90 min at 30 °C. After incubation, the reactions were terminated by adding SDS loading buffer, resolved by SDS-PAGE, followed by immunoblot with anti-ubiquitin and anti-Nedd4-1 antibodies. The addition of 40uM of 1BI3C to the reaction mixture significantly inhibits the auto-ubiquitination of Nedd4-1.

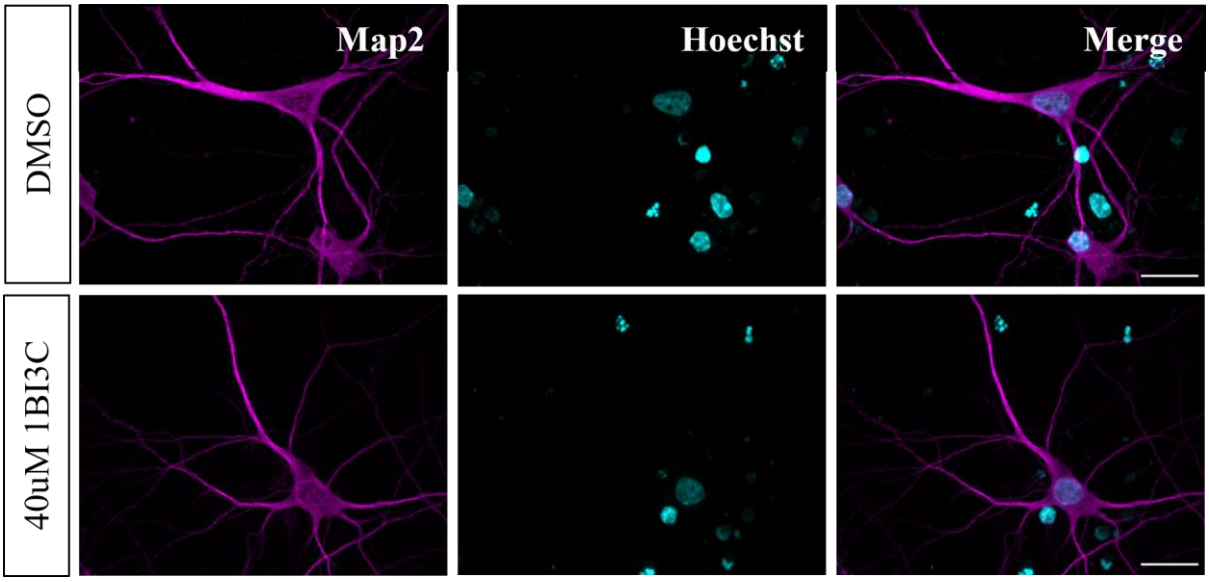

Figure 3.5. Cell health is not compromised after 24 hour 1-benzyl-indole-3-carbinol treatment. MAP2 immunostaining and Hoechst nuclear staining of dissociated hippocampal neurons after treating with 1BI3C for 24 hours. Scale bar 20um.

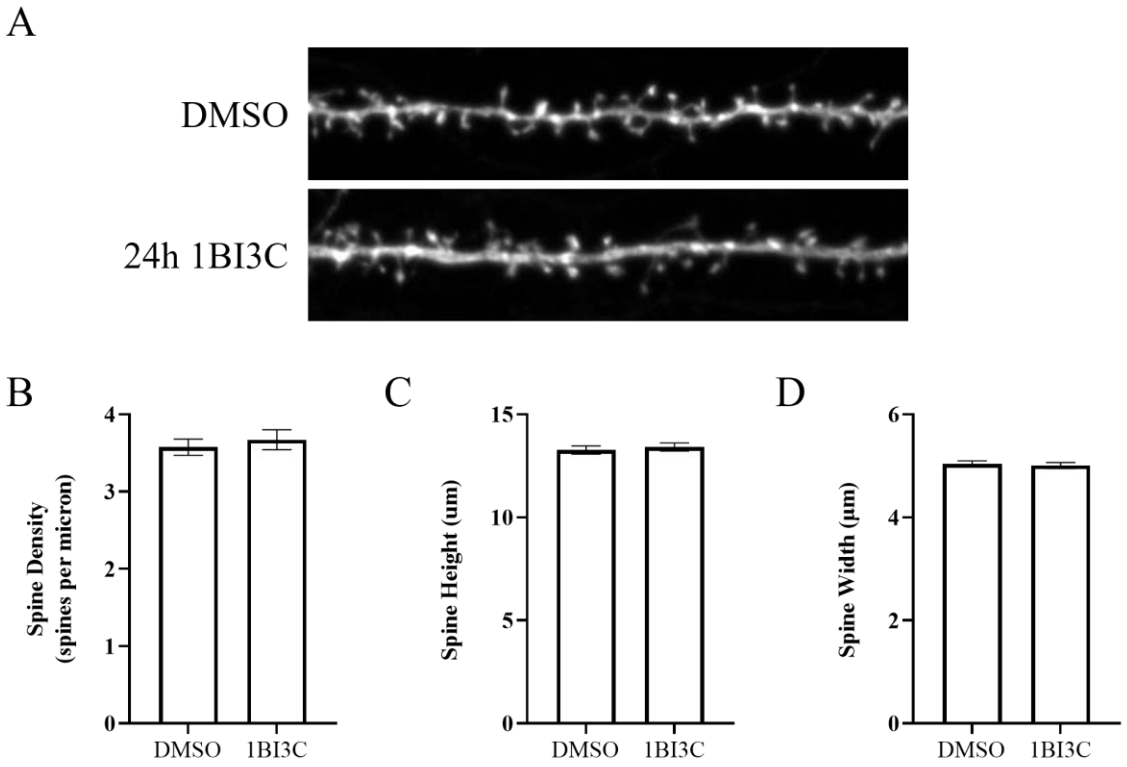

Figure 3.6. 40uM 1-benzyl-indole-3-carbinol treatment does not alter dendritic spine density. (**A**) DIV21 rat hippocampal neurons were infected with Sindbis GFP fluorescent fill and were treated with 40µM 1-benzyl-I3C or DMSO control for 24 hours. (*n* = 20 dendritic segments, 35µm long, per condition). (**B, C, D**) No significant difference was observed between spine density, spine height, or spine width between conditions (Unpaired t-test ^{ns}p = 0.5714, 0.6005, 0.7220, respectively).

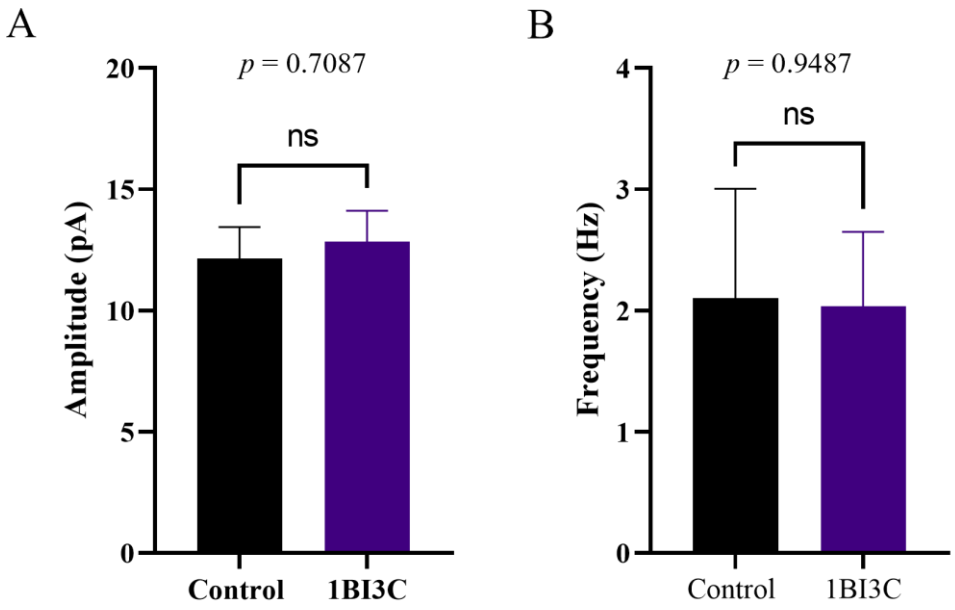

Figure 3.7. 24 hour incubation with 40uM 1-benzyl-indole-3-carbinol does not alter mEPSC amplitude or frequency. DIV21 dissociated hippocampal neurons were treated with DMSO or 1BI3C for 24 hours. We found no significant difference between the two conditions with respect to either mEPSC (**A**) amplitude (DMSO 12.1 ± 1.30 pA, 1BI3C 12.8 ± 1.27 pA, Unpaired t-test $^{ns}p = 0.7087$, $n = 6, 7$ neurons, respectively) or (**B**) frequency (DMSO 2.10 ± 0.900 Hz, 1BI3C 2.03 ± 0.614 Hz, Unpaired t-test $^{ns}p = 0.9487$, $n = 6, 7$ neurons, respectively).

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Chapter IV

Investigating Nedd4-1 function in two forms of NMDA-dependent synaptic plasticity

A. Abstract

The ubiquitin-proteasome system (UPS) is an important mediator of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)-type glutamate receptor (AMPAR) trafficking and degradation. It is known that Neural precursor cell-expressed, developmentally down-regulated protein 4-1 (Nedd4-1) contributes to the regulation of AMPARs by ubiquitinating the GluA1 subunit of AMPARs in an activity-dependent manner. Whether Nedd4-1 activity is required for the internalization of AMPARs that is observed in either GI-LTP Reset or NMDA-dependent chemical long-term depression (cLTD) has yet to be investigated. Here, by using the novel small molecule inhibitor of Nedd4-1, 1-benzyl-I3C (1BI3C), we show that inhibition of Nedd4-1 activity partially blocks the loss of surface-expressed AMPARs in GI-LTP dependent Reset in mixed cortical cultures. In contrast, 1BI3C does not significantly inhibit synaptic depression of NMDA cLTD in whole-cell recordings of hippocampal neurons under the 3minutes of 20uM paradigm that we used. Similarly, loss of Nedd4-1 in our KO model does not impair the expression of NMDA dependent cLTD. Overall, these data support our previous findings suggesting that Nedd4-1 dependent ubiquitination of AMPARs, leading to their internalization, is not activated by this method of direct activation of NMDA. However, this does not refute the notion that Nedd4-1 is involved in AMPAR trafficking subsequent to NMDA activation. NMDA signaling promotes phosphorylation, and receptor ubiquitination is enhanced with phosphorylation inhibitors, suggesting that there is a dynamic interplay between these two pathways that should be further investigated. Using our previously validated genetic and pharmacological tools, we

plan to further investigate the role that Nedd4-1 plays in intracellular sorting of receptors following NMDA-dependent synaptic cues.

B. Introduction

It is vitally important to brain function that neurons adapt flexibly and dynamically to incoming signals from partnering neurons. These dynamic responses to changes in network activity underly basal communication as well as learning-dependent changes, also known as synaptic plasticity. For example, neurons that receive sustained levels of activation over long periods of time may downregulate the cellular machinery responding to such signals. In other instances, neurons may receive signals that trigger mechanisms to enhance and amplify the reception of these inputs. Neurotransmitter-dependent communication and ion exchange at a chemical synapse is highly sophisticated and yet relies on a relatively small number of neurotransmitter receptors. This necessitates a tight temporal and spatial precision in the functioning of molecular processes and accessory proteins that control the identity and availability of surface receptor expression via exocytosis (membrane insertion) and endocytosis (removal from membrane) at the synapse. The molecular pathways regulating endocytosis alone are numerous, complex, and intertwined. Understanding the molecular mechanisms controlling this temporal and spatial precision will not only yield insights into standard brain function but will also uncover how these processes become dysfunctional in neuropathology and neurodegenerative diseases.

Two highly studied pathways that interface to control protein trafficking are the post-translational modification signaling mechanisms called phosphorylation and ubiquitination.

Phosphate or ubiquitin moieties are attached to proteins at very specific amino acid sites, and the presence or absence of these signaling molecules control the intracellular sorting and downstream fates of modified proteins. Neural precursor cell-expressed, developmentally down-regulated protein 4-1 (Nedd4-1), an E3 ubiquitin ligase, has been shown to ubiquitinate the AMPA-subtype of glutamatergic neurotransmitter receptors, (AMPARs), leading to AMPAR internalization in homeostatic downscaling as well as in acute agonist-induced internalization (Schwarz et al., 2010; Scudder et al., 2014). AMPARs mediate a majority of fast synaptic signaling and therefore their regulation is critically important for the neural plasticity underlying healthy brain function.

Phosphorylation and ubiquitination modifications coexist on AMPARs, and it is the subject of continual work to outline under what conditions they act as competitive or cooperative cues in different forms of synaptic plasticity. For instance, AMPARs are phosphorylated at 2 key positions within their C-terminal tail (serine residues number 831 and 845). AMPAR membrane stability is dependent, in part, on which of these two sites are phosphorylated. The dephosphorylation of site S845 on GluA1 is associated with receptor endocytosis and LTD mechanisms, sharing a functional overlap with ubiquitination in this regard, and this complex interplay calls for further investigation.

We think that the signaling pathways that engage phosphorylation also work in concert with ubiquitination pathways, but how and when these two processes converge has yet to be fully elucidated. We have found robust activity-dependent recruitment of Nedd4-1, ubiquitination of AMPARs, and AMPAR internalization in response to AMPAR stimulation, but the contribution of Nedd4-1 ubiquitination in response to NMDAR stimulation remains

elusive. Elucidating the direct interplay between ubiquitination and phosphorylation crosstalk in NMDA-dependent cLTD is a challenging question whose answers would give valuable insights into the mechanisms behind the phospho-ubiquitin axis as well as synaptic plasticity regulation as a whole. We began our probe into the characteristics of the NMDAR activation based phospho-ubiquitin module by investigating the impact of Nedd4-1 inhibition and knockout (KO) on two forms of NMDA-induced synaptic plasticity paradigms.

First, glycine-induced long-term potentiation (GI-LTP) is an agonist-induced form of synaptic plasticity in which the binding of glycine to the NMDA-subtype of glutamatergic neurotransmitter receptors (NMDARs) is shown to enhance postsynaptic AMPAR expression. GI-LTP leads to increased surface AMPARs, spine enlargement, and increased synaptic activity (Fortin et al., 2010; Jin et al., 2021; Li et al., 2016; Lu et al., 2001). Additionally, Fortin et al. found that LTP-dependent spine enlargement is dependent on the presence of calcium-permeable AMPARs (CP-AMPARs). Since CP-AMPARs play such a unique role in this form of synaptic plasticity, if Nedd4-1 is involved in the modification and internalization of GluA1 subunit-containing CP-AMPARs, it would highlight a new and novel function that Nedd4-1 plays in regulating synaptic plasticity. In order to study the role that Nedd4-1 plays in post-GI-LTP receptor maintenance, we monitored the surface expression and activity of AMPARs well after the LTP induction phase and found that AMPARs are indeed internalized following GI-LTP, as early as 20 minutes after their upregulation, and we refer to this phase of maintenance as the *Reset* phase.

Second, NMDA-dependent chemical long-term depression (cLTD) is another form of agonist-induced synaptic plasticity whereby the binding of NMDA to NMDA receptors

triggers intracellular processes leading to the dephosphorylation and internalization of AMPARs (Kameyama et al., 1998; Lee et al., 1998) as well as synapse loss and spine shrinkage (Henson et al., 2017). Though the time scale of internalization and depression is much faster in NMDA cLTD compared to GI-LTP Reset, it is thought that transient recruitment and incorporation of CP-AMPARs also underlies the signaling mechanisms leading to AMPAR internalization in cLTD (Sanderson et al., 2016). However, it has yet to be determined whether calcium influx through CP-AMPARs triggers endocytic mechanisms required for NMDA-dependent cLTD. Early work suggests that the influx of calcium through NMDAR activation leads to the activation of the phosphatase Calcineurin, dephosphorylation and internalization of AMPARs, and expression of LTD (Beattie et al., 2000), and so it is compelling to hypothesize that CP-AMPARs could share a function in these two synaptic plasticity paradigms, and that Nedd4-1 contributes to CP-AMPAR regulation.

Since both GI-LTP and NMDA-cLTD plasticities are thought to rely on the recruitment of CP-AMPARs, and since we know that Nedd4-1 ubiquitinates the GluA1 subunit of CP-AMPARs, we hypothesized that these synaptic signals may engage Nedd4-1 to ubiquitinate GluA1 for internalization. To address the role of Nedd4-1 in these two forms of endocytosis, we evaluated whether inhibiting Nedd4-1 enzymatic activity, via the small molecular inhibitor 1BI3C, or knocking out Nedd4-1 using a conditional knockout blocks either post-GI-LTP Reset or NMDA-induced cLTD, as measured by surface GluA1 expression and synaptic activity. In the following work we demonstrate that inhibition of Nedd4-1 activity using 1BI3C was sufficient to block the GI-LTP Reset we observed, suggesting that Nedd4-1 may be involved in molecular pathways downstream of NMDAR

activation. In contrast, we found that neither 1BI3C in dissociated rat neurons nor the Nedd4-1 conditional knockout in dissociated mouse neurons lead to significant changes in NMDA-induced depression. Probing Nedd4-1's activity under these synaptic plasticity paradigms will help us better understand the nuances of Nedd4-1 function and influence within these signaling pathways.

C. Materials and Methods

Primary Dissociated Cultures: Cortical tissue (outer cortex and hippocampus) were obtained from P1 homozygous Nedd4-1 floxed mice that recombines out the Nedd4-1 allele only with the addition of cre. Transgenic animals were kindly provided by Dr. Hiroshi Kawabe (Max Planck Institute). For rat preps, only hippocampal tissues was collected. Tissues were dissected from the brains of either sex pups in ice-cold dissection media and transferred to a 37° C water bath. Tissues were enzymatically digested using papain (Worthington Biochemical Co. LS003127) solution and subsequently rinsed in plating media (PM) and transferred to an enzyme-inactivating solution. The cells were then triturated in PM, centrifuged, resuspended, and plated on poly-D-lysine (Fisher Scientific 354210) coated coverslips or poly-D-lysine 6 well plastic dishes. Cultures were maintained in B27-supplemented Neurobasal media (Invitrogen) at 37° C, 5% CO_2, and 95% relative humidity until 14–21 days. AAV1-Hsyn-dsRed (1.61E+11 titer units/mL) and AAV2-CAG-mCherry-p2A-Cre 2.76E+12 titer units/mL (Salk GT3 Core) were diluted 1:10 and 1:50, respectively, and added directly to the culture media between DIV 7–10 and incubated for 7–14 days of infection. Rat neurons were used at DIV14-21.

Western blot analysis. Total protein lysates were generated by scraping cells into RIPA buffer (50 mM Tric-HCl, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors and incubated for 20 minutes, rotating at 4°C. Protein concentration was determined by BCA protein assay (Fisher Scientific 23225) and was normalize prior to sample buffer addition. Samples were boiled with sample buffer, resolved on 4-12%, Tris-Glycine gel (Invitrogen Novex Mini Protein Gel XP04122), transferred to nitrocellulose paper, and probed with primary antibodies. Blots were digitized and band intensities were quantified using Fiji (ImageJ). Band intensities were normalized to the band mean intensity of tubulin from each respective sample.

Immunocytochemistry: DIV14-18 neurons were washed with cold PBS-MC (phosphate buffered saline + magnesium and calcium) 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. Tables 1 and 2 list the primary and secondary antibodies used for immunocytochemistry, respectively. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 1 hour at room temperature. Coverslips were mounted onto glass slides for confocal imaging.

Primary and secondary antibodies. Map2 Ck 1:20000 Abcam Inc. ab5392; PSD95 Ms 1:1000 EMD Millipore CP35; Bassoon Ms 1:2000 Enzo VAM-PS003; Ms-488 Goat 1:1000 Life Technologies A11001; Rb-488 Goat 1:1000 Life Technologies A11034; Rt-488 Goat 1:1000 Fisher Scientific A11006; Ck-647 Goat 1:1000 Fisher Scientific A21449.

Confocal microscopy. All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nikon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 m/pixel at 1X), Plan Apochromat 40X/1.0 or 63X/1.0 numerical aperture objective, and an argon/krypton 100mW air-cooled laser for 488/568/647nm excitations. Maximum projected confocal Z-stacks were analyzed with ImageJ. Statistical significance was determined through unpaired t tests or ANOVA with specified post hoc multiple-comparisons test using Prism software (GraphPad).

Electrophysiology. Whole-cell patch-clamp recordings of miniature EPSCs (mEPSCs) were obtained from DIV14-16 dissociated mouse cortical neurons infected with either AAV-dsRed or AAV-mCherry-p2A-cre. Recordings were obtained at room temperature in a HEPES-buffered saline recording solution containing the following: 119mM NaCl, mM5 KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 30mM glucose, and 10mM HEPES, pH 7.2. The bath solution contained 1uM TTX (R&D Systems Inc. 1069), 10uM Bicuculline (R&D Systems, Inc. 0130), and 50uM D-APV (Tocris 0106). The electrode recording solution contained the following: 10mM CsCl, 105mM CsMeSO3, 0.5mM ATP, 0.3mM GTP, 10mM HEPES, 5mM glucose, 2mM $MgCl_2$, and 1mM EGTA, pH 7.2. Electrode resistances ranged from 2.5 to 4 MΩ and access resistances ranged from 5 to 20 MΩ. Cells were recorded at a holding potential of -70 mV with a calculated liquid junction potential of approximately 12.5mV. Signals were amplified, filtered to 2 or 5 kHz, and digitized at 10 kHz sampling frequency. mEPSCs were analyzed using ClampFit 10.3 (Molecular Devices) by averaging the amplitude and frequency of 150-300 events for each trace, with 5mV as the minimum threshold. Histograms were also generated for the collection of individual amplitudes and plotted as a cumulative probability.

Statistical analyses. Statistical differences between conditions were analyzed using either unpaired t tests (two groups) with indicated corrections for unequal variances or by ANOVA and indicated post hoc multiple-comparison test (>2 groups) conducted in Prism software (GraphPad).

D. Results

The increased expression of surface AMPARs resets to baseline levels following GI-LTP

We wanted to explore novel synaptic plasticity paradigms within which to further investigate the applications of pharmacological inhibition of Nedd4-1 and cre-dependent Nedd4-1 knockout. Since a variety of methods can induce an upregulation of AMPARs at the cell surface for temporary synaptic potentiation, or increase, in neural firing and communication, we reasoned that there would be a subsequent period of internalization in order to reset the receptor abundance and cell signaling back to a basal level. We postulated that GI-LTP, a form of synaptic plasticity that results in upregulation of surface AMPARs in response to glycine, would have a subsequent reset period in which the endocytic machinery, and possibly Nedd4-1, would be highly active.

We found that the increased population of surface expressed AMPARs are internalized as early as 20 minutes following LTP induction (Figure 4.2). We proceeded to call this phase *Reset* and evaluate the level of receptor abundance and activity during the reset period (ANOVA with Tukey's multiple comparisons test; Compared to Control (100%): GI-LTP 185 ± 32.4%, $*p = 0.015$, Reset 89.6 ± 28.1%, $*p = 0.011$; $n = 3$).

1-benzyl-I3C inhibits post-GI-LTP GluA1 internalization (Reset)

Using this framework, we tested whether 1BI3C blocks post-GI-LTP Reset. First, as expected, we found a significant increase in the surface expression of GluA1 under the GI-LTP treatment outlined (Figure 4.3. GI-LTP: 161 ± 16.9 percent of control, $*p = 0.022$), which resets back to or below baseline levels (Reset: 85.7 ± 14.1 percent of control, $**p = 0.004$). Curiously, when neurons are incubated with 1BI3C for 90 minutes prior to Reset, Reset is slightly, but insignificantly, impaired compared to Reset without 1BI3C (Reset + 1BI3C: 128 ± 14.5 percent of control, $^{ns}p = 0.136$, $n = 5$). The level of surface GluA1 under Reset + 1BI3C was not significantly different to any other condition, placing it at level between that of GI-LTP and Reset. This suggests but does not definitively demonstrate that Nedd4-1 may be involved in the later-stage Reset period of GluA1 internalization that follows GI-LTP. To evaluate the effects of GI-LTP, Reset, and the impacts of 1BI3C on Reset in single cells, we employed whole-cell electrophysiology.

1-benzyl-I3C does not significantly alter GI-LTP Reset decrease in mEPSC amplitude

We also observe a similar relationship in GI-LTP-dependent Reset when evaluating the amplitude of mEPSCs (Figure 4.4). There was a significant decrease in mEPSC amplitude during the Reset period of post-GI-LTP compared to control and GI-LTP (Control: 15.8 ± 1.15 pA, $n = 10$; GI-LTP: 19.0 ± 1.18 pA, $n = 7$; Reset: 11.0 ± 0.93 pA, $n = 5$; values represent mean ± SEM). When we treated neurons with the Nedd4-1 inhibitor 1BI3C, there appears to be a trend whereby 1BI3C may slightly reverse the effect of post-GI-LTP endocytosis, however, we are not able to detect a significant difference in this relationship

within the error of our experiments (Reset: 11.0 ± 0.930 pA; Reset + 1BI3C: 14.2 ± 0.811 pA, n = 9; ^{ns}p = 0.2264). Lastly, there was no significant differences in frequency between any conditions (ANOVA p = 0.9607), suggesting that GI-LTP did not impact presynaptic mechanisms or changes in post-synaptic cell spine number.

These data suggest that Nedd4-1 may be contributing to the post-glycine Reset but is complicated by the fact that in preliminary experiments, glycine treatment was not sufficient to drive Nedd4-1 recruitment to synapses during the reset period (data not shown). It is possible that we failed to capture a fast recruitment phase and that Nedd4-1 subsequently diffused back to steady-state levels at the time of fixation, or that the glycine conditions used were not sufficient to drive activation of the appropriate signaling pathways. For instance, a more deliberate inactivation of NMDARs, as seen in other GI-LTP work, may be required to provide the best signal to noise for determining Nedd4-1 recruitment. Further experiments are necessary to rule out these two possibilities.

1BI3C does not inhibit NMDA-dependent cLTD expression

Application of NMDA has been shown to cause a depression of synaptic activity in a variety of contexts. For example, in acute slices, 3 minutes of 20uM of bath-applied NMDA leads to dephosphorylation of GluA1 and significant depression of the evoked responses (Lee et al., 1998). When we apply 20uM of NMDA to neurons for 3 minutes and record after a 20 minute recovery period, we see a significant decrease in both amplitude and frequency of mEPSCs (Figure 4.4). Application of 1BI3C did not block the NMDA-induced synaptic depression, in either amplitude (Control: 17.1 ± 0.785 pA, n = 23 cells; NMDA: 14.4 ± 0.532

pA, n = 32 cells; NMDA + 1BI3C: 13.8 ± 0.772 pA, n = 24 cells; Fig. 4.4 B) or frequency (Control: 3.49 ± 0.438 Hz, n = 23 cells; NMDA: 2.4 ± 0.252 Hz, n = 32 cells; NMDA + 1BI3C: 1.9 ± 0.160 Hz, n = 24 cells; Fig. 4.4 B) of the whole-cell mEPSCs we recorded.

1BI3C does not inhibit NMDA-induced synapse pruning

In addition to the decrease in mEPSC amplitude and frequency we observe, we wanted to determine whether we see the characteristic decrease in synaptic contacts following NMDA stimulation as reported previously (Henson et al., 2017). This group also determined that while one application of NMDA was sufficient to induce the observed spine changes, two applications of NMDA was necessary to measure synapse loss more reliably via fluorescent markers and with more consistent synapse loss across individual dendrites. In our experiments, we also saw this robust NMDA-induced synapse loss (Figure 4.6), assessed by quantifying PSD95 puncta (Control: 45.2 ± 5.02 puncta; NMDA: 25.8 ± 3.41 puncta; NMDA + 1BI3C: 30.6 ± 2.66 puncta; n = 10 dendritic segments per condition). Compared to control, NMDA induced significant PSD95 loss (**p = 0.004), and NMDA + 1BI3C induced significant loss as well (*p = 0.034). There was no significant different between the NMDA treated and NMDA + 1BI3C treated dendrites (^{ns}p = 0.653), thus NMDA-induced PSD95 puncta loss was not blocked by 1BI3C.

Strikingly, in these experiments, filipodia-like synapses were significantly more prominent in NMDA conditions (data not shown) however, more work is necessary to determine whether this is due to an NMDA-induced shrinkage of existing spines, NMDA-induced promotion of new spine formation, or an experimental artifact.

NMDA-dependent cLTD is present in KO neurons

When we evaluated NMDA-induced cLTD in our knockout model, we found that NMDA treatment led to a significant depression of mEPSC amplitude in the knockout (KO 14.7 ± 0.982 pA; KO + NMDA 10.2 ± 0.663 pA; n = 12, 12; ANOVA with correction for multiple comparisons $^{**}p = 0.0048$). In these iterations of experiments, we saw a slight trend but no significant changes between WT and WT treated with NMDA, which likely requires a higher sample size to detect (WT 13.4 ± 0.982; WT NMDA 11.1 ± 0.683; n = 13, 10; ANOVA with correction for multiple comparisons $^{ns}p = 0.3415$). The trend of reduced amplitude does hold however, and further testing may reveal a depression of amplitude and frequency in both WT and KO under NMDA conditions. What our data does reveal is that the magnitude of depression was significantly stronger in KO neurons, and prompts further questions as to whether Nedd4-1 is acting on other key players within the NMDA-dependent synaptic plasticity signaling pathway. Further unanswered questions remain as to the phosphorylation and ubiquitination states of AMPARs in these contexts.

E. Discussion

Nedd4-1 is an essential regulator of many aspects of physiology and has the very striking characteristic of exhibiting a duality of functions under different contexts. For example, in the field of cancer biology, work has uncovered the role of Nedd4-1 as both a tumor suppressor and oncogenic promoter. This suggests that Nedd4-1 plays a variety of roles in very biological context-specific conditions. It is therefore critical to understand the precise

biological contexts that activate Nedd4-1 and how this can affect biological outcomes, in health and disease.

Our data reveal that in two forms of NMDAR-dependent synaptic plasticity, the internalization of AMPARs was not entirely abolished with Nedd4-1 inhibition or knockout. NMDA-dependent cLTD appears to operate independently to Nedd4-1 ubiquitination of AMPARs, supporting our previous evidence that NMDA activation did not lead to the recruitment of Nedd4-1 or ubiquitination of these receptors (Schwarz et al., 2010). Instead, the crosstalk between ubiquitination and phosphorylation of AMPARs in this context may be more nuanced.

Further, Nedd4-1 has many other targets with important functions, so it is possible that Nedd4-1 is engaging these other pathways orthogonally to its function in receptor internalization. For instance, it's been shown that NMDA-dependent cLTD occurs through the disassembling of the synaptic scaffold protein PSD95 through PTEN dependent phosphorylation of PSD95. Since PTEN is a known substrate of Nedd4-1, this begs the question of whether, in addition to activity-dependent ubiquitination of AMPARs, if Nedd4-1 also modifies and influences the activity of other protein pathway modulators. In our Nedd4-1 knockout, we observed that the magnitude of NMDA-dependent decrease in amplitude was stronger in the knockout neurons than in wild type, and we suspect that the absence of a PTEN negative regulator may enhance the depression, but further work needs to be carried out in order to verify this relationship. Needless to say, synaptic NMDA stimulation alone is not sufficient to drive the recruitment of Nedd4-1, and blocking Nedd4-1 catalytic activity does not interfere with NMDA-dependent cLTD.

Interestingly, in our previous work, we have found that glycine treatment recruited lysosomes to dendritic spines in an NMDA-dependent manner, and that blocking lysosomal degradation led to an increase in mEPSC amplitude (Goo et al., 2017). Lysosomes are important cellular machines that help maintain the integrity of the proteome by breaking down and recycling the components of misfolded, aggregated, or otherwise no longer needed proteins. We previously established that lysosomes can be recruited to synapses in an activity-dependent manner. Since GI-LTP drives the redistribution of lysosomes to spines, this suggests that synaptic activity may cue the intracellular space to prepare for lysosomal degradation following GI-LTP. Because we saw an intermediate effect of 1BI3C treatment on GI-LTP Reset, we hypothesize that there is a complex and dynamic interplay between the ubiquitination of AMPARs by Nedd4-1, and the phosphorylation of AMPARs, both in response to NMDA stimulation. There is clear motivation to pursue the investigation of how these two post-translational modification based molecular pathways converge to mediate activity-dependent receptor internalization. Additionally, we posit that Nedd4-1 could be interacting with other intracellular proteins that contribute to post-internalization sorting for lysosomal degradation in addition to its role in receptor internalization. For example, Nedd4-1 may interface with ESCRT to regulate endosomal sorting and degradative fate of these internalized AMPARs, following endocytosis.

Overall, these results provide further evidence that Nedd4-1 could be functioning outside of, or downstream to, the initial endocytosis of AMPARs. It is also possible that NMDA-dependent cLTD functions entirely independently of Nedd4-1, in which case this demonstrates high specificity for activation signals leading to Nedd4-1 ubiquitination of

AMPARs. Since ubiquitination of AMPARs does occur in cLTD, the E3 ligase responsible for ubiquitination of AMPARs following synaptic NMDAR activation needs to be discovered. Nedd4-1 could be regulating targets other than AMPARs to enhance internalization, or it could be responding to synaptic NMDA activation by strictly engaging the protein sorting pathways. We believe that there is a dynamic and complex interplay between ubiquitination and phosphorylation in NMDA cLTD that requires continued work to elucidate further.

F. Acknowledgments

Chapter 4, in part, is currently being prepared for submission for publication of the material. Hoffner, Nicole; Gilmore, Stephen, Dozier, Lara; Patrick Gentry N. The thesis author was the primary investigator and author of this material.

G. Figures and Tables

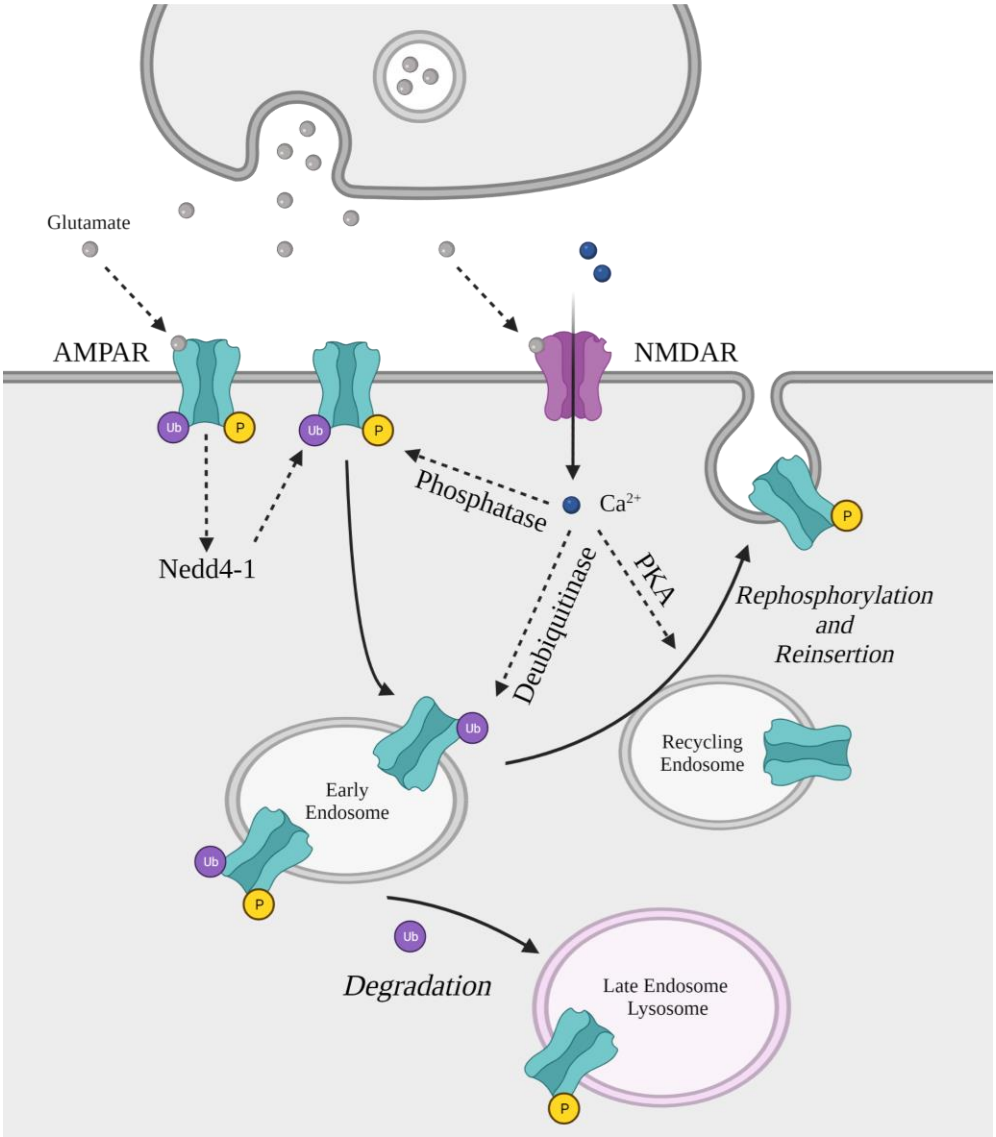

Figure 4.1. Nedd4-1 dependent ubiquitination contributes to protein internalization, sorting, and degradative pathways, in combination with phosphorylation pathway crosstalk. Nedd4-1 is recruited to synapses in response to AMPAR activation. There, it ubiquitinates AMPARs, which also undergo dephosphorylation processing. Following internalization, receptors are sorted for either recycling back to the membrane or for degradation via the endo-lysosomal system.

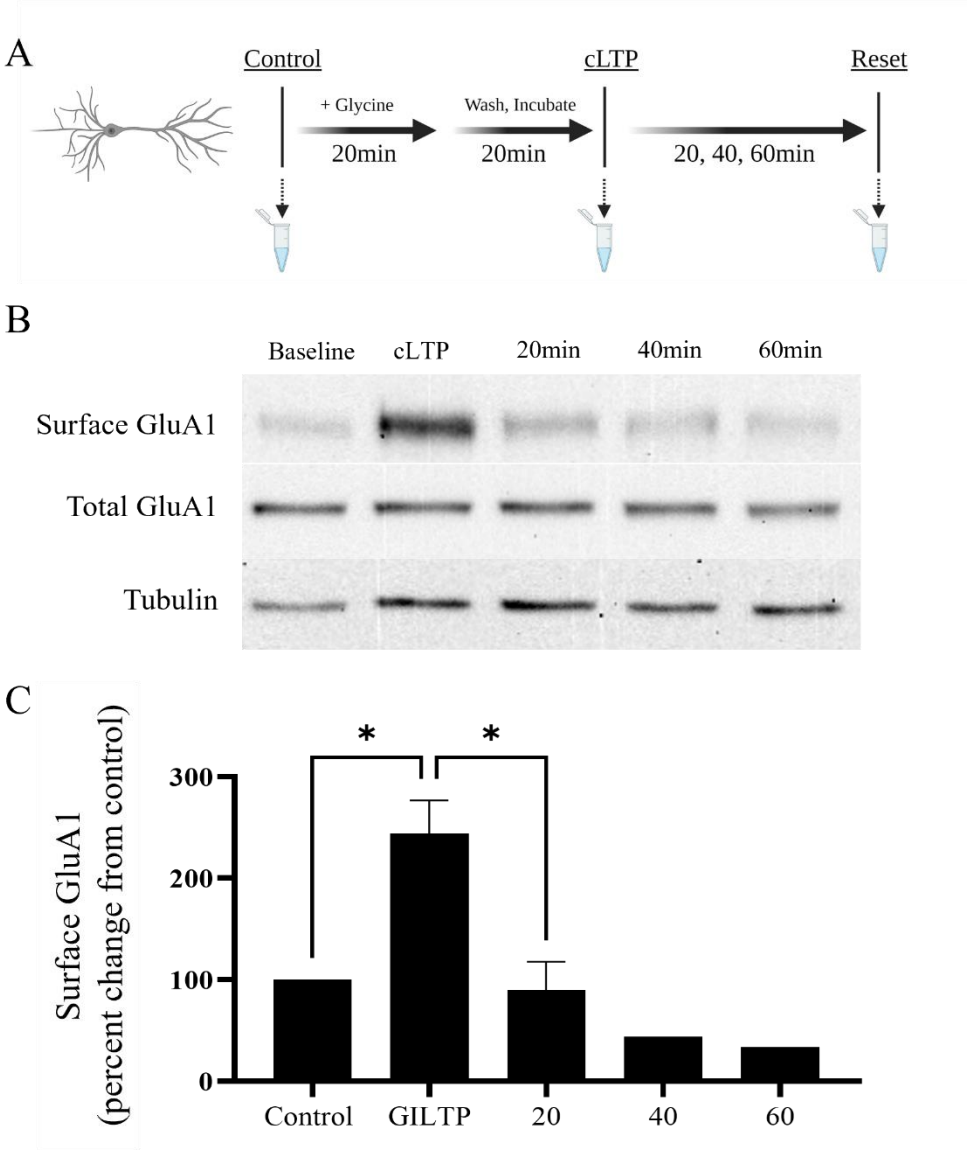

Figure 4.2. GI-LTP induced surface receptor increase resets to baseline levels after 20 minutes. (A) Schematic diagram illustrating the experimental method. GI-LTP was induced in cultured cortical neurons by incubating cells with 200uM glycine for 20 minute followed by a 20 minute incubation after wash-out. This 40 minute period was sufficient to induce a robust increase in surface expression of AMPA receptors (GI-LTP) followed by an eventual internalization phase, called Reset. (**B**) Western blot showing that surface GluA1 level increased with the 40 minute glycine and wash treatment, followed by robust internalization 20 minutes following the GI-LTP protocol. Total levels of GluA1 remained the same over the course of the experiment. (**C**) Quantification of the signal in B, graph shows mean ± SEM, ANOVA followed by Tukey's multiple comparison test, $n = 3$, $^*p < 0.05$.

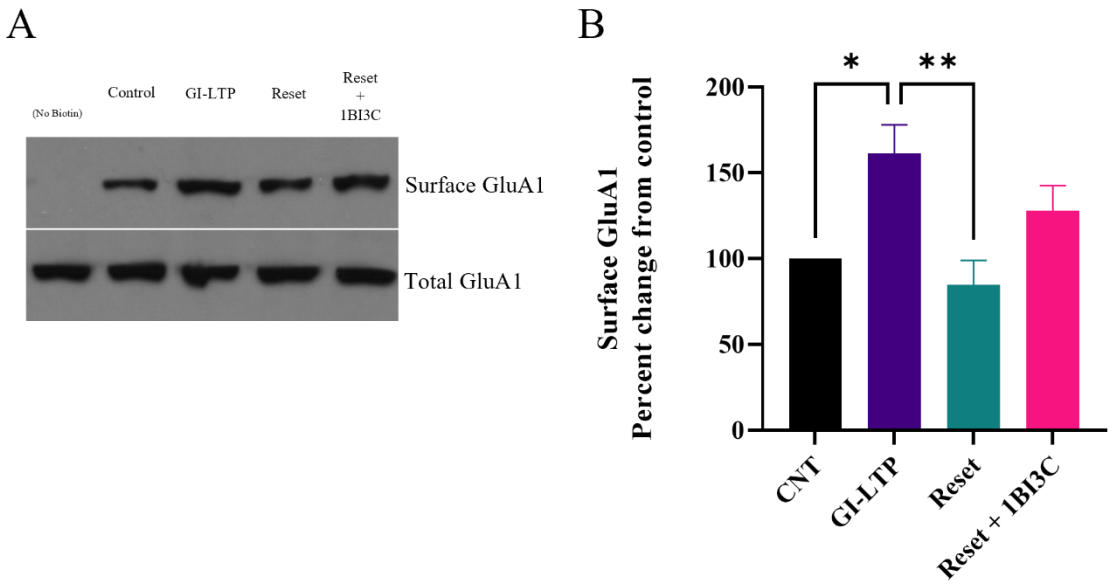

Figure 4.3. 1BI3C inhibits GI-LTP induced surface GluA1 Reset. (**A**) Representative blot showing protein abundance in GI-LTP and Reset (**B**) Quantification of band intensity shows an increase in the surface expression of GluA1 under the GI-LTP protocol (GI-LTP: 161 ± 16.9 percent of control, *p = 0.022), which resets back to or below baseline levels (Reset: 85.7 ± 14.1 percent of control, **p = 0.004). Pretreatment with 1BI3C leads to Reset surface levels of GluA1 that are not significantly different from either control, LTP, or Reset.

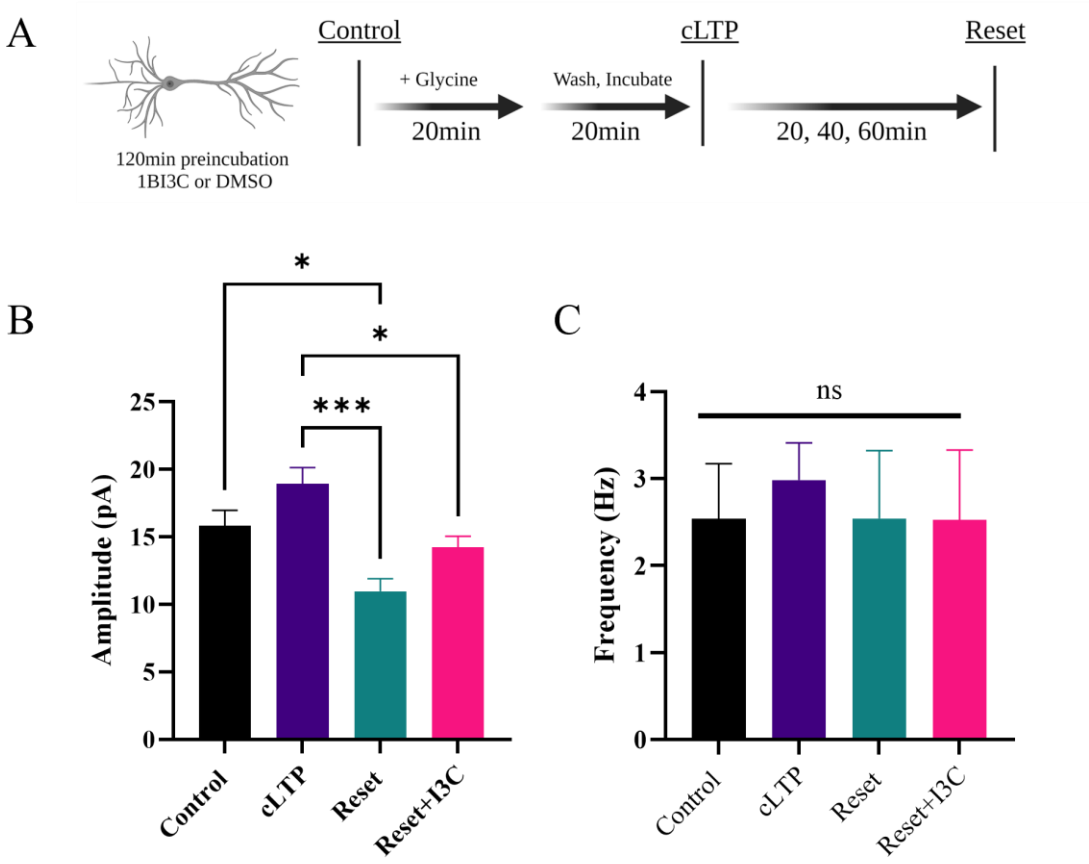

Figure 4.4. 1BI3C does not block GI-LTP dependent decrease in mEPSC amplitude. (**A**) Timing of drug treatment in GI-LTP. DIV18 –21 neurons were treated with 200uM glycine for 20 minute followed by a 20 minute incubation after wash-out to allow for upregulation of surface AMPARs. 1BI3C condition was also treated with a 2 hour pre-treatment of 40uM 1-benzyl-I3C (1BI3C) (**B**) Quantification of mEPSC amplitude showing a significant decrease in mEPSC amplitude during the Reset period of post-GI-LTP compared to control and GI-LTP (Control: 15.8 ± 1.15 pA, n = 10; GI-LTP: 19.0 ± 1.18 pA, n = 7; Reset: 11.0 ± 0.93 pA, n = 5; values represent mean ± SEM). When we treated neurons with the Nedd4-1 inhibitor 1BI3C, there appears to be a trend whereby 1BI3C may slightly reverse the effect of post-GI-LTP endocytosis, however, we are not able to detect a significant difference in this relationship within the error of our experiments (Reset: 11.0 ± 0.930 pA; Reset + 1BI3C: 14.2 ± 0.811 pA, n = 9; ^{ns}p = 0.2264). (**C**) We observed no significant differences in frequency between any conditions (ANOVA ^{ns}p = 0.9607)

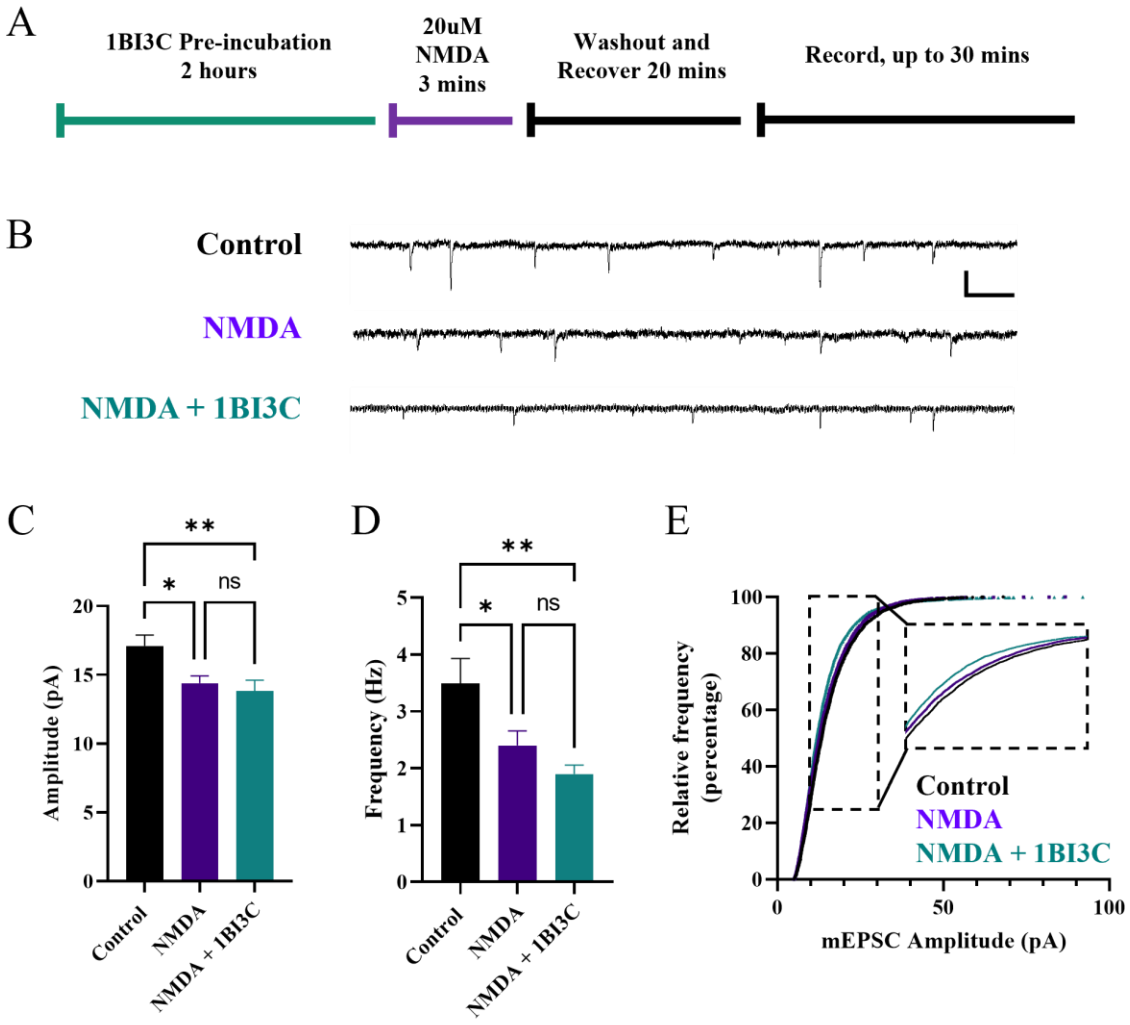

Figure 4.5. Inhibition of Nedd4-1 does not block NMDA-dependent cLTD. (**A**) Timing of drug treatment in NMDA-induced synapse loss. DIV18 –21 neurons were treated for 3 minutes with 20uM NMDA, with or without a 2 hour pre-treatment of 40uM 1-benzyl-I3C (1BI3C) (**B**) Representative traces of mEPSCs recorded from dissociated hippocampal neurons. Scale bar, 10 pA and 200 ms. (**C, D**) Quantification of mEPSC amplitude (C) and frequency (D) in all three conditions, demonstrating a decrease in mEPSC amplitude and frequency that is not blocked by 1BI3C inhibition of Nedd4-1 activity; graphs show mean ± SEM, ANOVA followed by Tukey's multiple comparison test, *$p < 0.05$, **$p < 0.01$, n = 23-32 cells over 5 independent experiments. (**E**) Cumulative probability distributions of all mEPSC amplitudes recorded from Control, NMDA, NMDA + I3C treated neurons. n = 3272, 3278, and 5286 events, respectively.

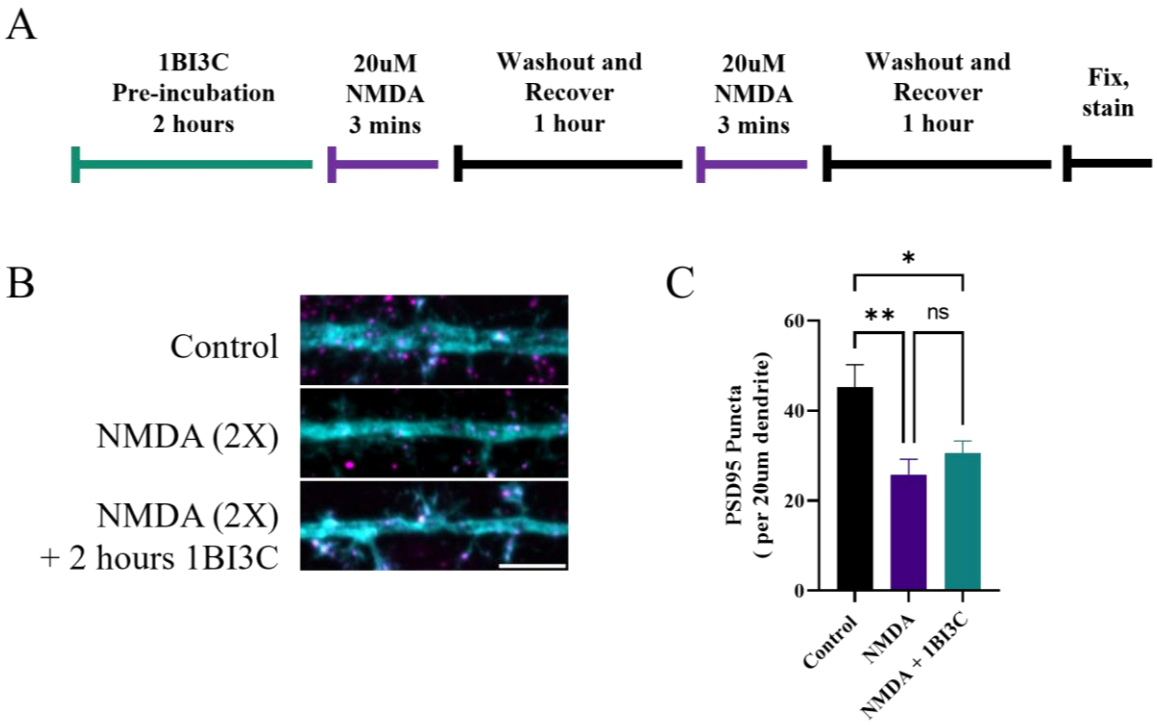

Figure 4.6. NMDA-induced synapse elimination is not reversed by 1BI3C. (**A**) Timing of drug treatment in NMDA-induced synapse loss. DIV21 neurons were treated for 3 minutes with 20uM NMDA before washing out drug and letting them recover for 1 hour. This process was repeated once more before fixing and staining. (**B**) Representative images of 20um dendritic shaft segments of neurons treated with DMSO, NMDA, or NMDA + 1BI3C. (**C**) Quantification of synapse density (total number of synapses per 20um segment), showing that NMDA-induced loss of PSD95 puncta is not reversed with 1BI3C treatment (Control: 45.2 ± 5.02 puncta; NMDA: 25.8 ± 3.41 puncta; NMDA + 1BI3C: 30.6 ± 2.66 puncta). Graph shows mean ± SEM, ANOVA followed by Tukey's multiple comparison test, *$p < 0.05$, **$p < 0.01$, $n = 10$ dendritic segments per condition. Scale bar 5um.

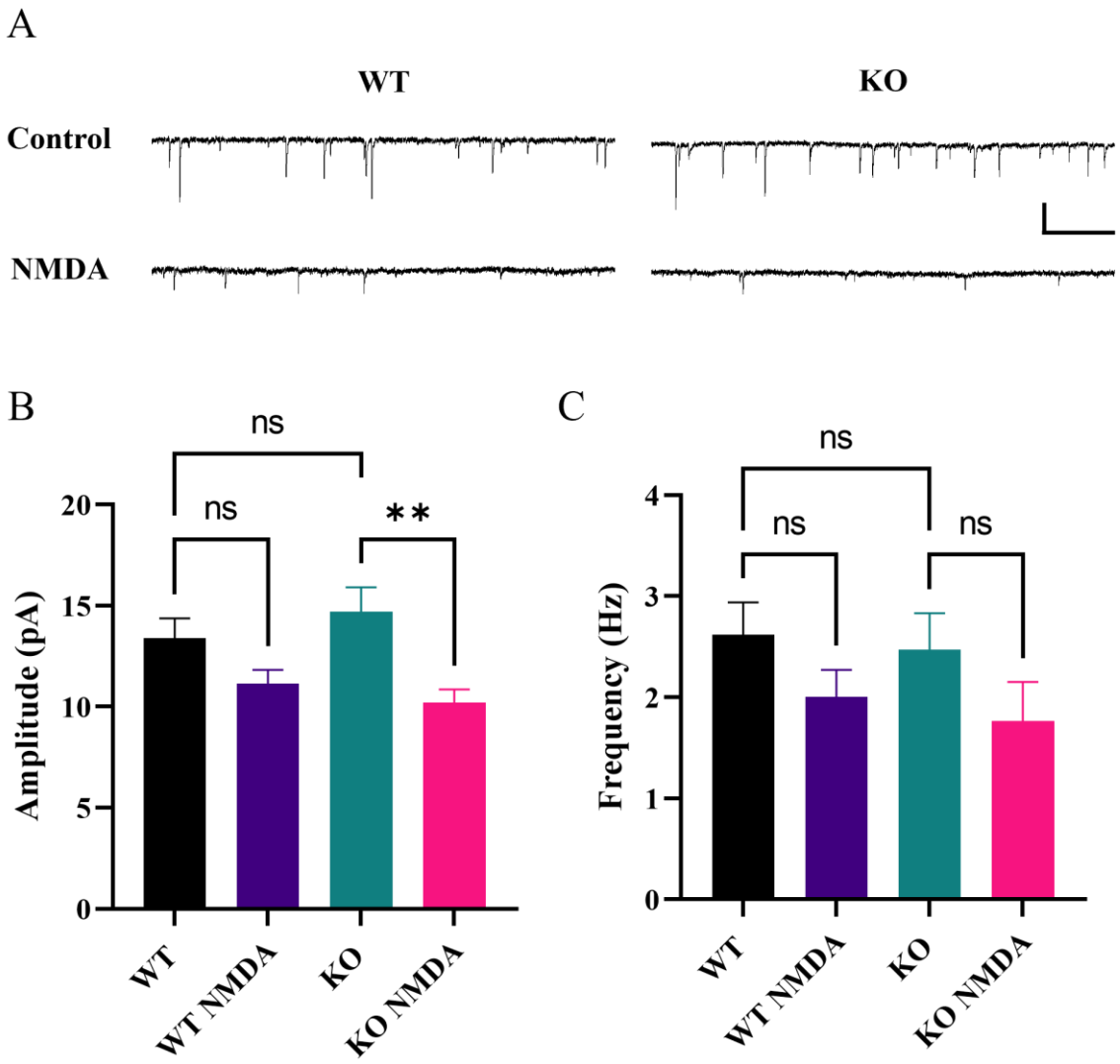

Figure 4.7. Nedd4-1 neurons express NMDA-dependent cLTD. (**A**) Representative traces of mEPSCs recorded from dissociated neurons DIV14-18, treated for 3 minutes with 20uM NMDA. Scale bar, 500 ms and 20 pA. (**B, C**) Quantification of mEPSC amplitude (B) and frequency (C) demonstrating a decrease in mEPSC amplitude and frequency in both WT and KO; graphs shows mean ± SEM, ANOVA followed by Tukey's multiple comparison test, $^{**}p < 0.01$, n = 10-13 cells over 3 independent experiments.

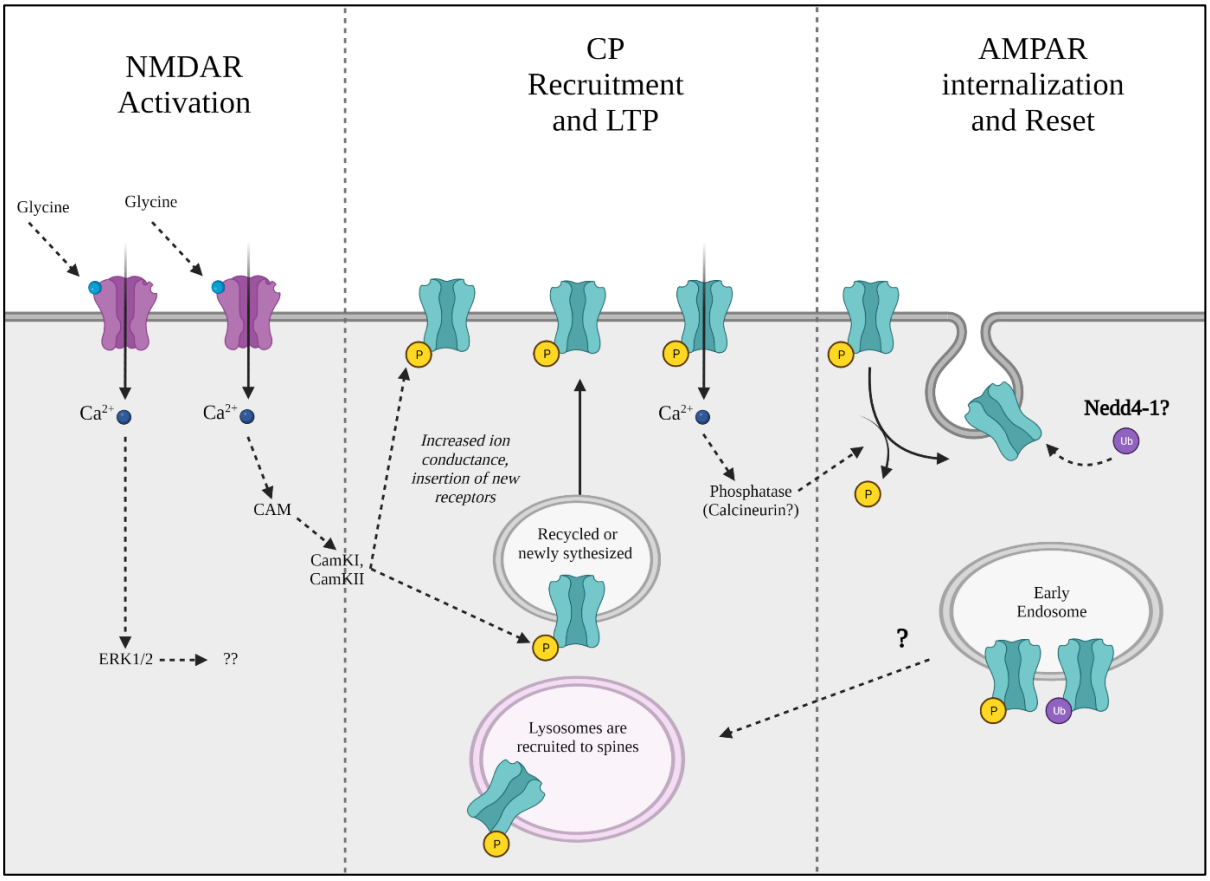

Figure 4.8. Model depicting the involvement of Nedd4-1 dependent AMPAR trafficking in GI-LTP and Reset. GI-LTP triggers upregulation of surface AMPARs via agonist-induced synaptic plasticity. Glycine binding to the NMDA-subtype of glutamatergic neurotransmitter receptors (NMDARs) enhances postsynaptic AMPAR activity, leading to increased surface AMPARs, spine enlargement, and increased synaptic activity. GI-LTP also drives the redistribution of lysosomes to spines. GI-LTP occurs through calcium activation of protein kinases leading to the phosphorylation of AMPARs, whether through ionotropic or metabotropic NMDAR activation. The recruitment of CP-AMPARs enhances LTP-dependent spine enlargement and potentiation. As early as 20 minutes following LTP, AMPARs are internalized in the *Reset* phase.

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Chapter V

Conclusion

The benefits arising from further elucidating the role of Nedd4-1 in regulating the synaptic plasticity of neurotransmitter receptors are manyfold. First, while the field has made significant progress in determining the identity and functions of Nedd4-1 substrates, a complete interactome has yet to be defined. Identifying new substrates through advanced techniques and technologies will open innumerable avenues of scientific inquiry. Additionally, upstream of Nedd4-1 ubiquitination of substrates, and as a substrate of itself through auto-ubiquitination, understanding the functional consequences of protein-protein interactions regulating the activation of Nedd4-1 will contribute to the monumental task of unraveling the complexities of dense molecular signaling networks. Furthermore, identifying the exact synaptic activation cues leading to downstream Nedd4-1 recruitment and ubiquitination of its substrates will advance our understanding of the precision of the molecular mechanisms involved in receptor maintenance and synaptic plasticity expression. Also, since dynamic expression of synaptic plasticity underlies neural communication more broadly, through neural circuitry regulating adaptive learning, memory encoding, motor activity, sensory reception, etc., understanding how these miniscule molecular processes contribute to more higher-level circuit functions provides a fuller picture of overall brain function and behavior. Finally, given that these molecular processes are so critical for proper brain function, shedding light on the dysregulation of molecular processes, such as impairments to Nedd4-1 dependent ubiquitination of neurotransmitter receptors, will provide new understandings of how Nedd4-1 activity (or lack of) may lead to chronic illnesses and neurodegenerative diseases.

A. Tools for future studies of Nedd4-1 function in mature neurons

We have demonstrated in this body of work that we can culture mature neurons from mice expressing a homozygously expressed floxed Nedd4-1 alleles. Nedd4-1 is recombined out in a cre recombinase-mediated fashion, leading to significantly reduced expression of Nedd4-1 protein, thereby facilitating a genetic knockout of Nedd4-1. We observed that delaying the cre-mediated knocking out of Nedd4-1 allowed the neurons to develop without significant impairments to dendritic arborization, spine formation, or synapse expression. Removing Nedd4-1 from the cells after early development enables us to assess the effects of Nedd4-1 loss in mature neurons without complications arising from Nedd4-1's functions in early development. This provides us with an accessible model in which to study Nedd4-1's unique role in neurotransmitter receptor removal, sorting, and degradation.

Additionally, we demonstrated that the small molecule inhibitor 1-benzyl-indole-3-carbinol (1BI3C) is also a promising tool for investigating Nedd4-1 function. Because pharmacological interventions and drug treatments can be performed on much smaller timescales than a genetic knockout (minutes and hours compared to days and weeks) this provides us with a complementary toolset to investigate Nedd4-1 function in a wide variety of molecular environments and contexts. Pharmacological inhibition confers the advantage of highlighting the effects of impaired Nedd4-1 activity by activity directly on the enzyme to inhibit its function, while the knockout provides the advantage of a more physiologically relevant model of Nedd4-1 impairment. Both provide new and necessary information to understanding the role of Nedd4-1 in the modification and regulation of protein substrates,

and combined, will provide a much needed framework for interrogating the complexity of molecular signaling networks.

B. Nedd4-1 regulation of neurotransmitter receptors

Alpha-amino-3-hydroxy-5-methyl-4-isoxaolepropionic acid (AMPA)-type glutamate receptors mediate the majority of fast excitatory synaptic transmission in the nervous system, giving rise to neuronal communication between cells, within and between circuits and brain regions, and thus overall brain function. Therefore, the dynamic changes to AMPA receptor availability for neuronal signaling requires a high degree of temporal and spatial fidelity in the trafficking of AMPARs to and from the synapse. Small perturbances to the temporal and spatial dynamics of trafficking gives rise to impairments in neural activity, leading to impairments in learning and memory. Further, changes to the spatial expression of AMPARs in the synapse can lead to dysfunction and disease (Henley & Wilkinson, 2016).

Subcellular localization of AMPAR type and number within a synapse is a dynamic process in which AMPARs are delivered to and subsequently removed from the postsynaptic membrane in response to a multitude of molecular signals, and on timescales ranging from seconds to days. After removal of these receptors, they can be recycled and delivered back to the membrane or sorted into the endo-lysosomal pathway that leads to their ultimate degradation (Goo et al., 2015; Schwarz et al., 2010; Scudder et al., 2014). Understanding how AMPARs are processed for endosomal sorting and lysosomal degradation is as of yet incompletely understood. Many questions remain as to what synaptic cues trigger the molecular pathways leading to sorting, what protein modifications promote sorting, what are

the combinatorial effects of protein modifications on receptor processing for recycling or degradation, and where in the network of protein modification does the ubiquitin ligase Nedd4-1 impart its activity-dependent regulation of AMPARs? Asking these and other important questions related to the function of Nedd4-1 in the brain will require the newly adapted genetic and pharmacological tools we have previously outlined, in addition to novel and advanced technologies that uncover the fast temporal and spatial dynamics of activation cues and functional outcomes.

C. Elucidating the role of Nedd4-1 in NMDA-dependent cLTD

The driving factors underlying synaptic plasticity include the dynamic insertion and removal of neurotransmitter receptors into the postsynaptic membrane. These up and down regulation processes give rise to long-term potentiation (LTP), long-term depression (LTD), as well as homeostatic plasticity. Neural activity at a synapse is a causal force in promoting these forms of responsive synaptic plasticity, and understanding which stimulation paradigms activate trafficking pathways is a core point of inquiry when investigating the molecular mechanisms underlying neural communication.

We evaluated the effect of Nedd4-1 knockout and inhibition on two forms of NMDA-dependent LTD: GI-LTP Reset and NMDA-cLTD. Our data revealed that in both of these forms of NMDAR-dependent synaptic plasticity, the internalization of AMPARs was not entirely abolished. In GI-LTP Reset, the intermediate level of AMPAR internalization blockade suggests there are contributing factors to the Nedd4-1 dependent internalization in this context, and this necessitates a model with better signal to noise. One approach includes

manipulating the phosphorylation states on the GluA1 subunit of AMPARs, eg by using a mouse model in which S845 cannot by phosphorylated, in order to isolate the effects of ubiquitination and downstream trafficking. We believe that these non-phosphorylatable receptors will be continuously delivered and removed from the membrane, followed by their degradation, and that using this approach will aid future experiments to investigate the crosstalk between ubiquitination and phosphorylation modification of AMPARs and the effect of combinatorial modifications in the context of synaptic plasticity.

Protein modifications such as phosphorylation and ubiquitination act as molecular determinants for intracellular processes such as the recycling or degradation of internalized AMPARs. Further elucidating the precise combinatorial functions, in response to dynamic synaptic cues, that these determinants carry out in their nuanced regulation of intracellular processes will lead to a better understanding of the molecular networks that go awry in disease, and therefore possible points of intervention.

D. Defining the role of Nedd4-1 in the trafficking of calcium-permeable AMPARs

A noteworthy substrate of Nedd4-1 is the AMPAR sub-type of homomeric GluA1, GluA2-lacking, calcium-permeable AMPARs. Because calcium plays such a pivotal role as a secondary messenger, involved in cellular processes including neuronal synaptic transmission, GPCR signaling pathways, and as a co-factor in some enzymatic reactions (including Nedd4-1's ubiquitination of substrates), ion channels that allow calcium to pass through the cell

membrane therefore play a key role in regulating these foundational molecular pathways. These calcium-permeable AMPARs (CP-AMPARs) have been shown to be involved in many forms of LTP expression (Clem & Huganir, 2010; Guire et al., 2008; Kelly et al., 2009; Lalanne et al., 2018; Lante et al., 2011; Man, 2011; P. Park et al., 2016; Pojeong Park et al., 2019; Plant et al., 2006; Tóth & McBain, 1998). Since CP-AMPARs are comprised of only GluA1 subunits, and Nedd4-1 ubiquitinates GluA1, not A2, 3, or 4, it is conceivable that Nedd4-1 plays an important role in the regulation of this receptor subtype. Our lab has previously demonstrated that Nedd4-1 can be recruited to spines in response to synaptic activation, on the time scale of minutes, and determining whether this phenomenon can be elicited in response to the accumulation of CP-AMPARs during LTP will provide immense insight into further refining our understanding of Nedd4-1 function in varying forms of synaptic plasticity. In this work, we have begun to address questions of the specificity of Nedd4-1 on the internalization of CP-AMPARs following LTP in our GI-LTP Reset framework, but evaluating the changes in isolated populations of CP-AMPARs is not trivial. Elucidating the regulation of isolated CP-AMPARs, in contrast to total AMPARs, requires methods that isolate the CP-AMPAR activity signal. It would be most beneficial to use, for example, a cobalt-calcein assay in which cobalt entry through CP-AMPARs and not GluA2-containing AMPARs quenches a fluorescent signal inside the cell, or employ inhibitors with a high selectivity for CP-AMPARs over other receptors, in combination with electrophysiology, in order to isolate the CP-AMPAR component, or electrophysiologically recording the current-voltage profile, or IV plot, to determine any changes in surface expression of the inwardly rectifying CP-AMPARs.

E. Uncovering Nedd4-1 function within the phospho-ubiquitin regulatory network

While many questions related to the crosstalk between phosphorylation and ubiquitination remain unanswered, both of these modifications are present on GluA1 and thought to regulate interactions with AMPAR trafficking mechanisms, controlling their synaptic insertion and removal. Bringing to light the specific contexts in which these two post-translational modifications act as competitive or cooperative cues to regulate synaptic plasticity will bring significant progress to the field. Since AMPARs undergo phosphorylation at different amino acid sites to regulate the receptors in different ways (eg membrane stabilization vs internalization), questions related to how ubiquitination interacts with these phospho states to enhance or inhibit molecular functions is of great interest. For instance, do AMPARs experience ubiquitination in their membrane-stabilized state? Are AMPARs dually modified in the initiation of internalization following synaptic plasticity, or is it a step wise function? Does the presence of phosphorylation enhance or inhibit Nedd4-1's ability to ubiquitinate GluA1 at the surface? Are phosphorylation and ubiquitination modifications competitive or cooperative signals for the sorting of internalized AMPARs? What is the timescale of Nedd4-1's role in the AMPAR resetting phase of internalization following an LTP upregulation of GluA1? We hypothesize that phosphorylation and ubiquitination pathways work in concert to regulate the trafficking of AMPARs, but how and when these two processes converge has yet to be fully understood. Advancing our understanding of the direct interplay between ubiquitination and phosphorylation crosstalk in different LTP and

LTD paradigms is a challenging question whose answers will provide valuable contributions to outlining and understanding the mechanisms behind the phospho-ubiquitin axis and how these molecular regulatory pathways control synaptic plasticity and brain function in a complex, combinatorial manner.

F. Refining our understanding Nedd4-1 function in neurodegeneration

Understanding the way in which Nedd4-1 function and dysfunction gives rise to disease states holds promising therapeutic potential in multiple areas of research. In addition to the field of oncology, in which Nedd4-1 has been shown to play both oncogenic and tumor suppressor roles (Jung et al., 2013; Wang et al., 2007; Yim et al., 2009; Zeng et al., 2014; Zhou et al., 2014; Zou et al., 2015), Nedd4-1 has been implicated in a variety of ways in neurodegenerative diseases such as Alzheimer's disease (AD) (Alrosan et al., 2019; Rodrigues et al., 2016), Parkinson's disease (PD) (Canal et al., 2016), and amyotrophic lateral sclerosis (ALS) (Nagpal et al., 2012). Many studies have linked chronic AMPAR dysregulation to disease progression in AD, ALS, and Huntington's disease (HD). In ALS, it is hypothesized that the high expression of calcium-permeable AMPA receptors leaves motor neurons more susceptible to glutamate toxicity and cell death (Carriedo et al., 1996), and interestingly, AMPA receptor antagonists have been shown to provide neuroprotective effects in animal models of PD, ALS, and ischemic stroke (Loschmann, 1991; Niu et al., 2018; Turski et al., 1998; Van Damme et al., 2003).

In line with the subunit-specificity in the trafficking and regulation of AMPARs, in addition to the implication of CP-AMPARs in disease, recent work has demonstrated a regulatory role of Alzheimer's risk factor CALM on CP-AMPARs specifically, in which the clathrin assembly lymphoid myeloid leukemia protein (CALM) regulates the surface levels of CP-AMPARs (Azarnia Tehran et al., 2022). Therefore, the influence of CALM in AD could be contributing to the physiological AMPAR dysregulation and glutamate toxicity seen in AD. It would be intriguing to probe for Nedd4-1 interaction in this context.

G. Characterizing the interactome profile of Nedd-1 and identifying non-AMPAR substrates that Nedd4-1 regulates in an activity-dependent manner

Finally, it is clear that Nedd4-1 plays potential roles in homeostatic cellular maintenance and disease, so it is of particular interest to uncover the complex network of Nedd4-1 interactions in order to understand its wide influence in neurophysiology. What proteins are activating Nedd4-1 for activity-dependent redistribution to the synapse? We know that synaptic activity leads to the mobilization of Nedd4-1 and its redistribution to the synapse, however the specific regulators of Nedd4-1 itself remain a mystery. Nedd4-1 itself is subject to both phosphorylation and ubiquitination modifications and understanding the kinases and phosphatases that regulate its activity is of significant importance. Knowing the regulators Nedd4-1 will help tease apart and illuminate the upstream and downstream activity-dependent processes occurring within these dense molecular networks.

In-vivo proximity labeling, utilizing APEX2 labeling and mass spectrometric quantification, is a very exciting and promising method to determine the interactome of Nedd4-1. It will shed light on the identity of proteins regulating Nedd4-1 as well as novel substrates of Nedd4-1 as of yet undiscovered. A few candidate proteins in the phosphorylation pathway and the endocytic sorting pathways that might be uncovered and that I would like to highlight here include the endosomal sorting complexes required for transport (ESCRT) pathway, the phosphatase and tensin homolog (PTEN), and protein kinase C (PKC).

First, the ESCRT pathway is a cellular mechanism used to manipulate plasma membranes into small cytosolic vesicles that help transport protein cargo to different subcellular locations. For instance, when ubiquitin-tagged proteins need to be removed from the cell membrane and shuttled to a lysosome for degradation, it is ESCRT complexes that coordinate the budding off of membrane pieces into a multivesicular body (MVB) that can move within the cell, translocating from the cell surface to the lysosome within the cell cytosol. ESCRT-0, the first complex in the process of chain reaction events leading to downstream protein sorting and degradation, specifically complexes with ubiquitinated proteins at the surface of the cell, promoting the initial steps preceding internalization. It mediates the transfer of tagged proteins from the synaptic membrane to the MVB membrane for intracellular shuttling. Given the spatial and functional proximity of ESCRT-0 and Nedd4-1, it will be enlightening to find whether they are responsive to the same molecular cues, and whether they function in response to similar synaptic plasticity paradigms.

Second, PTEN is a known substrate of Nedd4-1 and is known to be involved in the NMDA-dependent regulation of PSD95 leading to synaptic depression (Jurado et al., 2010).

In our experiments, we saw no reversal of th expression of synaptic depression in response to NMDA stimulation using either 1BI3C inhibition or the Nedd4-1 knockout, however, qualitative observation shows that the response to cLTD of both knockout cells and pharmacologically inhibited cells exhibits a very tight distribution of response. It is possible that if Nedd4-1 is a negative regulator of PTEN in this NMDA-stimulated cLTD, that we would find an enhancement of cLTD expression, which is similar to what we have observed, instead of the blockade of depression that we had hypothesized. However, measuring a decrease in amplitude of a signal that may be already saturated to the lowest measurable plateau will prove to be difficult and require a novel tool or approach.

Lastly, there is strong evidence to suggest that Nedd4-1 and PKC may overlap in the regulatory pathways involved in synaptic function. The PKC gain-of-function (GOF) variant PKCα M489V exhibits an increase in enzymatic activity (~30%) and leads to robust changes in the phosphoproteome of the cell and synaptic plasticity in neurons: characteristic changes in the brains of mice carrying this PKC variant include a susceptibility to amyloid-ß induced synaptic depression and a significant reduction in dendritic spine density (Lorden et al., 2021). This suggests that PKC is involved in synaptic depression, but at which regulatory nodes? Does its activity lead to downstream activation of Nedd4-1 function? Does increased PKC activity lead to reduced receptor expression? Or impact ubiquitin-dependent synaptic plasticity expression? Answering these and other questions related to the regulation of Nedd4-1 itself and the regulation of protein substrates by Nedd4-1 would yield significant advances in the field of molecular neurobiology.

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