UNIVERSITY OF CALIFORNIA SAN DIEGO

Characterizing the Trafficking and Function of Nedd4-1 in the Neuron

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in

Neurosciences

by

Kevin Lamont White Jr.

Committee in charge:

Professor Gentry Patrick, Chair
Professor Brenda Bloodgood
Professor Steve Briggs
Professor Gulcin Pekkurnaz
Professor JoAnn Trejo

2023

The dissertation of Kevin Lamont White Jr. is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

I dedicate this publication…

To my wife, Dywanyé Alicia Washington. Just by being herself, she constantly inspires me beyond anything I could dream of for myself. She is a bright light in my world. My life was forever changed when I met her. This PhD is as much hers as it is mine.

To my mother, Lorraine Graise White for filling my life with fierce and unyielding love. For teaching me to fight for myself and reach for everything I've wanted. For being a wonderful example to follow in my life

To my father, Kevin Lamont White Sr. A man who has been an unshakeable symbol of growth and respect in my life. For believing in my goals before I even knew what they were. For having a seemingly endless well of faith.

To my brother, Aaren Lamar White. For constantly bringing infectious joy into my life. For holding me accountable to my potential. I try everyday to lead a life full of authenticity the way my brother does.

To my sister, Lauren Nechelle White. For always being a source of comfort and joy in my life. For leading your life fearlessly and exuding excellence. I try to embodied your spirit in my life everyday.

EPIGRAPH

nobody exists on purpose

nobody belongs anywhere

we're all going to die…

come watch TV.

- Morty Smith, *Rick and Morty*

What happens to a dream deferred?

Does it dry up

like a raisin in the sun?

Or fester like a sore—

And then run?

Does it stink like rotten meat?

Or crust and sugar over—

like a syrupy sweet?

Maybe it just sags

like a heavy load.

Or does it explode?

- Langston Hughes, *Harlem*

TABLE OF CONTENTS

LIST OF FIGURES

Chapter III

Chapter IV

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Dr. Gentry Patrick for all of his support and guidance throughout my thesis program. I would like to thank my thesis committee members Dr. Joan Trejo and Dr. Steve Briggs for their wise words. I'd like to give special thanks to my committee members Dr. Gulcin Pekkurnaz and Dr. Brenda Bloodgood who both have been supporting me and looking out for me since the beginning of my time here at UCSD. They have given guidance in personal, scientific and professional aspects of my life and I appreciate them for it. I would also like to thank my undergraduate mentor Dr. Kevin Jones. He was the first to believe that I had what it takes to succeed in a graduate program. He was the first to teach me how to engage with science and the wonders of the brain. He took a chance on me before anyone else did.

I would like to give thanks to my friends and family throughout the years. A special thanks goes to my chosen family Dr. Chandler Puritty, Karen Valerio, and Jordan Campbell. These amazing people built me up and supported me in some of my hardest moments. I could not have continued in this program without them. I would like thank all of the friends I made in the Black Grad program. The community we built and the amazing memories we made will be etched into my mind for decades to come. Thank you for accepting me. I would like to thank my labmates and friends Dr. Natasha Slepak and Dr. Nicole Hoffner. Without them I would be lost. They gave science guidance, friendship, and made the lab space a place I called home. Thank you to Steven Gillmore and Lara Dozier for being consistent source of guidance and help throughout the years. Both of these wonderful scientists paved the way for my success in

many ways. I would also like to thank Carmella Villegas for being a wonderful undergraduate researcher and a huge help in the lab. She always brought positive energy and an impressive level of competency to every day in the lab.

Lastly I would like to thank the UCSD neuroscience graduate program for all of their support over the last seven years. In particular I would like to thank Erin Gilbert and Linh Pham. They went above and beyond to support in anyway necessary. I could not have gotten this without their tireless work and support.

Chapter 2, in whole, is currently being prepared for submission for publication of the material. Kevin White; Patrick Gentry N. The dissertation author was the primary investigator and author of this material.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Kevin White; Hoffner, Nicole, Gilmore, Stephen, Dozier, Lara; Patrick Gentry N. The dissertation author was the primary investigator and author of this material.

Chapter 4's work was supported by the The Sanford Burnham Prebys Proteomics Core. Thank you to Dr. Gene Yeo for providing the NES-APEX2-GFP used to clone our APEX2-HA-Nedd4-1 constructs. Thank you to Dr. Kim Dore's lab for contributing electrophysiological recordings using Nedd4-1 inhibitor, 1B-I3C.

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

VITA

2016	Bachelors of Science, Biology, Howard University, Washington, DC, USA
2016 – 2019	UC-HBCU Fellowship Award, University of California San Diego, La Jolla, CA, USA
2016 – Present	Co-Founder of the Colors of the Brain, Minority Students Mentorship Organization, University of California San Diego, La Jolla, CA, USA
2017 – 2021	Principal Member of the Black Graduate and Professional Student Association University of California, La Jolla, CA, USA
2020	Neuroscience Graduate Teaching Award, University of California San Diego, La Jolla, CA, USA
2023	Doctor of Philosophy, Neurosciences, University of California San Diego, La Jolla, CA, USA

FIELDS OF STUDY

Major Field: Neurosciences

Studies in Cellular Neurobiology
Professor Gentry Patrick

ABSTRACT OF THE DISSERTATION

Characterizing the Trafficking and Function of Nedd4-1 in the Neuron

by

Kevin Lamont White Jr.

Doctor of Philosophy in Neurosciences

University of California San Diego, 2023

Professor Gentry Patrick, Chair

The purpose of this dissertation is to bring a greater understanding of the trafficking and function of the ubiquitin E3 ligase, neuronal expressed downregulated in development protein 4-1 (Nedd4-1), in the neuron.

Neuronal cell health depends on the delicate regulation of the various protein abundances across the neuron through protein homeostasis (proteostasis). Proteostasis consists of three major parts: protein synthesis, maintenance, and degradation. Ubiquitination

by the E3 ligase plays an important part in proteostasis by targeting proteins for degradation. In this dissertation, I focus on studying how Nedd4-1's trafficking and protein interactions are tightly controlled by synaptic activity. These studies help better elucidate Nedd4-1's normal function in neurons and implications in neurological disease.

In Chapter II, I present a comprehensive review of Nedd4-1's described role in the central nervous system (CNS) in health and disease. This provides context for the experimental questions posed throughout this thesis. In Chapter III, we sought to understand rules regulating Nedd4-1 trafficking in neurons. Because cellular compartments differ greatly, subcellular localization of proteins is often finely regulated and intimately tied to their function. We created a GFP-tagged Nedd4-1 expression construct for use in live time-lapse imaging in hippocampal neurons to study the trafficking dynamics of Nedd4-1. First, we validated the utility of GFP-Nedd4-1 in live imaging. Next, we show that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor activation drive distinct trafficking dynamics of GFP-Nedd4-1 in neurons. Together these data suggest Nedd4-1's trafficking is subject to fine and distinct activity-dependent regulation in neurons.

In Chapter IV, we further explored Nedd4-1's function in neurons by using APEX2 mediated proximity labeling to characterize Nedd4-1's interactome. We created and validated the utility of APEX2-HA-Nedd4-1 expression and proximity labeling in primary neuronal cultures. We show that in untreated neuronal cultures, APEX2-Nedd4-1 had a significant association with spliceosome complex components and mRNA processing pathways. In contrast, NMDAR activation significantly increased APEX2-Nedd4-1 interaction with

synaptic proteins. Using co-localization with postsynaptic marker PSD-95 and pharmacological inhibition of Nedd4-1 we show that NMDARs recruit Nedd4-1 to the synapse to possibly participate in regulation of GluA1 containing AMPARs there.

Chapter I

Introduction

A. Ionotropic glutamate receptors are crucial for synaptic health and function

The brain is a beautiful organ. The human nervous system is able to absorb sensory cues from its environment and use those cues to adapt for success. The adult human brain is outfitted with approximately 90 billion neurons that you carry with you throughout most of your life. A majority of your brain does not receive new neurons so the ability of your brain to plasticly adapt to its environment lies in the thousands to tens of thousands of connections – synapses – each neuron makes. Neurons exhibit a miraculous ability to delicately modulate the strength at each synapse across their cell membrane. Plasticity of the brain arises from this strength modulation. This basic function of the neuron is essential to a healthy brain and disruption of this process is a hallmark of many neurological disorders. If we can understand a neurons ability to carefully modulate synaptic strength, we will have uncovered the building blocks that underlie cognition in the brain as well as bring light to the pathophysiology of many neurological diseases.

Synaptic modulation can happen locally at individual synapses, Hebbian plasticity (Huganir RL, et al 2013), or more globally across the neuron, homeostatic scaling (Turrigiano GG, 2008). In both scenarios, the strength of the synaptic connection is largely determined on the postsynaptic side by the number of ionotropic receptors present at the surface to receive neurotransmitters. There are three major types of glutamatergic receptors present at the surface: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)

receptor, the N-methyl-D-aspartate (NMDA) receptor, and the kainate receptor. Our work focuses on AMPA and NMDA receptors. The AMPA receptor is a type of ionotropic glutamate receptor (iGluR) that mediates a majority of the fast excitatory transmission in neurons. Gating kinetics of the AMPAR allow for signaling on the milliseconds time scale compared to other iGluRs, like the NMDA receptor, which respond on a time scale of tens to thousands of milliseconds (Traynelis SF, et al. 2014). The number of AMPA receptors present at the synapse is a good estimate for its synaptic strength. For this reason, there is a large breath of literature studying the regulation and function of AMPA receptors trafficking to and from the synapse. Due to its slower kinetics, the presence of NMDA receptors at the synapse are not as directly correlated to synaptic strength as AMPA receptors, but they still serve a crucial role for synaptic plasticity. The NMDA receptor's permeability to calcium (Ca^{2+}), diversity of subunit composition, and involvement in a wide range of downstream signaling pathways make it invaluable to plasticity at the synapse and even the regulation of AMPA receptors. To uncover the mechanisms driving synaptic plasticity, a further understanding of both iGluR types and their interplay is needed.

To understand the NMDA receptor's role in synaptic plasticity it is important to explore how the functionality of the NMDA receptor arises from its diversity in structure. NMDA receptors are formulated of four different subunits (Paoletti P. et al, 2013). There are seven possible NMDA receptor subunits: GluN1 subunits, GluN2(A-D), and GluN3(A and B) (Traynelis SF. Et al, 2014; Cull-Candy SG. et al, 2004; Paoletti P. et al, 2011). A dimer of two GluN1 subunits serves as the base of every type of NMDA receptor configuration. The variability arises from the different combinations of GluN2 and GluN3 subunits. Di-

heteromeric NMDA receptors consist of one GluN1 dimer in combination with a GluN2A, GluN2B, GluN2C, GluN2D, or GluN3A dimer. Tri-heteromeric NMDA receptors consist of one GluN1 dimer and a combination of two of the other six possible subunits. Most of the variety amongst these subunits comes from the cytosolic C-terminal end. These differences lead to changes in permeation, gating properties, pharmacology, and interaction in downstream pathways. In addition to this, NMDA subunit composition shows changes due to brain region and maturation of neurons.

When it comes to NMDA receptors' role in synaptic plasticity, the GluN2 subunits seem to be most heavily involved. Many early studies used genetic manipulation and pharmacological perturbation to describe a dichotomy between GluN2A and GluN2B whereas the former was crucial to the long-term potentiation (LTP) at the synapse and the latter important for long term depression (LTD) (Sakimura K. et al, 1995; Brigman JL. Et al, 2010). In contrast, a number of following studies suggest that both GluN2A and GluN2B are essential for LTP and not necessarily LTD. A prevailing theory in the field is that LTP requires a partnership between di-heteromeric (GluN1 / GluN2A) receptors and tri-heteromeric (GluN1 / GluN2A / GluN2B) receptors to drive insertion of AMPA receptors (AMPARs). The GluN2A's increased permeability to Ca^{2+} and GluN2B C-terminal domain's affinity for Calcium–calmodulin (CaM)-dependent protein kinase II (CaMKII) – a kinase known to play an important role in the trafficking of AMPA receptors – work in concert to drive LTP at the synapse (Hardingham G et al, 2019).

Like the NMDA receptor, AMPA receptor structure plays a huge role in their trafficking turnover at the synapse. AMPA receptors exist as heterodimers built up of four possible

subunits, GluA1-4. GluA1/2 and GluA2/3 dimer pairs represent a majority of the AMPA receptor make-up in the hippocampus. GluA1 homomers represent a minority and GluA4 subunits are seen most often during development and not so much in the mature neurons (Diering, GH. et al, 2018). Each of these subunits has distinct structures and biophysical characteristics that dictate the function of the AMPA receptor. Whereas all AMPA receptors are permeable to Na^{+}, GluA2 lacking AMPA receptors (GluA1 homomers and GluA1/3 dimers) are also Ca^{2+} permeable. This is important to note not only because Ca^{2+} plays a huge role as a second messenger and numerous metabolic pathways throughout the cell, but it is also suggested in the field that Ca^{2+}-permeable AMPA receptors (CP-AMPARs) play a distinct role in synaptic plasticity or synaptic dysfunction but also may play a role in regulating the trafficking and turnover of Ca^{2+}-impermeable AMPA receptors (Man HY. et al, 2018).

The biophysical properties of AMPARs play a big role in their trafficking to and from the synapse but another important regulator of AMPAR trafficking is the posttranslational modification (PTM) of these receptors and the complex matrix of auxiliary proteins associated with the receptor. AMPARs can be modified by a suite of PTMs (phosphorylation, ubiquitination, SUMOlyation, etc.) all of which influence the targeting of the AMPAR to different cellular pathways. One of the more well studied of these PTMs is phosphorylation. AMPARs are typically phosphorylated on their intracellular C-terminal tail at specific serine, threonine, or tyrosine sites. Each receptor subunit can be phosphorylated at different sites which regulate varying features of synapse plasticity. Phosphorylation of the GluA1 subunit at S845 by PKA and S818 and S831 by CaMKII and PKC have all been associated with long

term potentiation (LTP) (Diering, GH. et al, 2018). Phosphorylation of the GluA2 subunit at S880 by PKC and Y876 by Src family kinase has been implicated in long term depression (LTD) and endocytosis of AMPARs from the synaptic membrane. Another PTM known to regulate AMPAR trafficking is ubiquitination. In fact, the first evidence for ubiquitination of mammalian AMPAR was discovered by the Patrick lab in 2010 (Schwarz, 2010). Below, we will further discuss the ubiquitination of the AMPARs and its auxiliary proteins and how it plays a crucial role in the trafficking of the receptor and the mechanisms defining synaptic plasticity throughout the central nervous system.

B. Nedd4-1's Role in synaptic function

Mammalian cells play host to tens of thousands of proteins of various abundances. Each of these proteins serve particular roles to contribute to the overall health and function of the cell. Protein homeostasis is the process by which the cell maintains optimal protein levels. This process is especially important in neuronal cells that must respond to changing external stimuli and therefore have dynamic proteomic demands. Protein homeostasis can be broken into two main parts: synthesis and degradation. Protein degradation is mediated in large part through the ubiquitin system and the proteasomal or endo-lysosomal systems. Many ubiquitin ligases target aggregated or misfolded proteins for degradation through the proteasome or lysosome. When protein homeostasis is disrupted, synthesis or degradation, there begins to be either a deficit in necessary functional proteins or a build-up of misfolded and aggregated proteins. In both cases dysregulation of protein homeostasis may often lead to toxicity in the

cell and underlies the pathophysiology of many neurological disorders (Hipp MS. et al, 2019; Malgaroli A. et al, 2006).

This thesis will be focusing on understanding the protein degradation arm of protein homeostasis and the rules and players regulating degradation in neurons. Ubiquitin is a 76-residue molecule that serves many functions in the cell. Ubiquitin is covalently attached to lysine residues on protein substrates and target them for degradation or many non-proteolytic pathways (change in activity, localization, affinity to binding partners, etc). Ubiquitination is mediated by three types of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligating enzymes. E1 activating enzyme group forms a thiol ester bond with the ubiquitin molecule thereby activating its C-terminal. The E2 conjugating enzyme carries the active ubiquitin either to its substrate or an E3 ligase. Then the E3 ligase catalyzes the binding of ubiquitin to its substrate (Pickart CM. et al, 2001). Attaching a single ubiquitin molecule to a substrate is called monoubiquitination and directs a substrate to specific downstream pathways. But ubiquitin tags can themselves be ubiquitinated at a single site to form polyubiquitin chains or at multiple sites to form branched polyubiquitinated chains (Akutsu M. et al, 2016). These monoubiquitin tags or polyubiquitin chains can also be modified by posttranslational modifications (phosphorylation, acetylation, SUMOylation). All of these ubiquitin modifications – polyubiquitin chain length, branching points, modification – make up what is known in the literature as the “ubiquitin code” and play a role in determining the fate of the substrate (Swatek KN. et al, 2016). As the field progresses, we will begin to decipher the hierarchy of this ubiquitin code and begin to form a

full understanding of the ubiquitin's role in regulating protein function, localization, and degradation.

Although ubiquitin's role in protein degradation has been known since the early 90s, it wasn't until the early 2000s (Colledge M. et al 2003) that ubiquitin was first described playing a role in the downregulation of AMPARs. A majority of the early work suggests ubiquitination of AMPAR auxiliary and anchoring proteins – like postsynaptic density protein 95 (PSD-95) (Colledge M. et al, 2003; Bingol B. et al, 2004), glutamate receptor interacting protein 1 (GRIP1) (Guo L. et al, 2007) – or ubiquitin mediated disruption of AMPAR interaction with scaffolding proteins – like Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1 (MAGI-1) (Emtage L. et al, 2009). Only a few studies suggested that direct ubiquitination of AMPARs is important for its downregulation (Patrick G. et al, 2003; Schaefer H. et al, 2006). Since then, literature on ubiquitin's role in AMPAR turnover and degradation has substantially expanded. A number of E3 ligases (Cortese G. et al 2016; Kim J. et al 2019; Ghilarducci K. et al 2021; Ma P. et al 2022; Pavlopoulos E. et al 2011) as well as deubiquitinating enzymes (DUBs) (Huo Y. et al, 2017) have been implicated in regulating the ubiquitination of AMPARs and modulation of synaptic transmission. Activity-dependent ubiquitination helps regulate the endocytic sorting of these receptors away from recycling (back to the membrane surface) and instead towards the proteasome/lysosome (Schwarz LA. et al, 2010; Widagdo J. et al, 2015). The Anggono lab has also suggested in multiple studies a crosstalk between posttranslational modifications by demonstrating how phosphorylation promotes GluA2 receptor subunit ubiquitination (Widagdo J. et al 2020) but attenuates GluA1 receptor subunit ubiquitination (Guntupalli S. et al 2017). This ubiquitin-

dependent downregulation of AMPA receptors also plays a role in the pathophysiology of many neurological disorders such as Alzheimer's Disease (AD) (Schwarz et al, 2010; Rodrigues EM. et al, 2018; Guntupalli S. et al, 2017; Zhang Y. et al, 2018; Azarnia Tehran D. et al, 2022) and Parkinson's Disease (PD) (Zhu M. et al, 2018; Cortese G. et al, 2016).

The Patrick lab was the first to reveal E3 ligase, neuronal expressed downregulated in development protein 4-1 (Nedd4-1)'s, role in GluA1 subunit ubiquitination and degradation. (Schwarz LA. et al, 2010) showed that AMPAR-dependent ubiquitination of AMPARs and their targeting to the lysosome is mediated by Nedd4-1. Removing Nedd4-1 occluded activity-dependent ubiquitination of the receptor. (Lin A. et al, 2011) confirmed these results. Our lab followed this study up with another observing that not only was Nedd4-1 responsible for mediating AMPAR-dependent ubiquitination of AMPA receptors, but that the Nedd4-1 protein clusters and colocalizes with the synapse in an activity dependent manner (Scudder et al 2014). This study also shows that Nedd4-1 trafficking to the synapse is calcium dependent. A calcium chelator reduced puncta formation and removal of the calcium binding domain of Nedd4-1 occluded redistribution of the ligase all together. When synapses are activated by light-stimulation you see an accumulation of Nedd4-1 at those synapses as well as an increase in AMPAR ubiquitination (Hou Q. et al, 2011). This ubiquitination mediated by Nedd4-1 seems to engage in cross talk with the phosphorylation of the serine 845 site on the C-terminal tail of GluA1. (Guntupalli S. et al, 2017) show that phospho-mimetic S845 reduces the ability of Nedd4-1 to bind and ubiquitinate GluA1. Nedd4-1 has also been shown to play a crucial role in AD through its regulation of functional AMPA receptors. Nedd4-1 silencing rescued Amyloid-beta (Aβ) treated neurons that typically show reduction in GluA1 surface receptor

levels, and mini-excitatory postsynaptic currents (Rodrigues E. et al 2016). AD brain lysates and Aβ treated neurons also show an upregulation of Nedd4-1 protein (Zhang Y. et al 2018). Although it is clear that Nedd4-1 plays a crucial part in regulating synaptic transmission through the AMPAR ubiquitination, there is still much to be understood about the cellular and biological mechanisms of this process.

C. A review of Nedd4-1's role in the CNS in health and disease

In Chapter II, presents a comprehensive summary of Nedd4-1's role in the central nervous system in health and disease. Nedd4-1 is incredibly important to the healthy function of neurons throughout the CNS and has been implicated in many studies to be involved in the pathophysiology of numerous neurological disorders. This section begins by discussing the history of Nedd4-1, how it was discovered, and its original described function. I will also detail the structure of Nedd4-1 as a way to give context to the following discussion about its role throughout the CNS. Then, I will discuss Nedd4-1's various roles in healthy functioning neurons. I discuss Nedd4-1's involvement in everything from axon branching to ionotropic receptor trafficking to the underlying mechanisms of drug action in the brain. I will also detail behavioral results of complete and partial ablation of Nedd4-1 in the brain. Finally, I will detail Nedd4-1's role in different neurodegenerative diseases, psychiatric disorders, cancer, ischemia, and neuropathic pain.

D. Glutamatergic signaling drives differing Nedd4-1 trafficking dynamics

Chapter III tackles the issue of how Nedd4-1 trafficking is regulated in neurons. Like many other proteins, Nedd4-1's subcellular localization greatly affects it function in the neuron. This section details the trafficking dynamics of Nedd4-1 and how they are differentially regulated under AMPA- or NMDA- dependent stimulation. We created a GFP-tagged Nedd4-1 viral-based expression construct for use in live time-lapse imaging in hippocampal neurons to study the trafficking dynamics of Nedd4-1. First, we validated the utility of GFP-Nedd4-1 in live time-lapse imaging in neurons and showed for the first time that Nedd4-1 trafficking in dendrites and recruitment to synapses is regulated by NMDAR activation. Furthermore, we show that AMPAR and NMDAR activation drive distinct trafficking dynamics of GFP-Nedd4-1 in neurons. GFP-Nedd4-1 puncta induced by NMDAR activation are smaller and less mobile than those induced by AMPAR activation. Together these data suggest Nedd4-1's trafficking, and thus function, is subject to fine and distinct activity-dependent regulation in neurons. Moreover, these data constitute the first direct evidence of an E3 ubiquitin ligase being regulated by activity-dependent trafficking in neurons.

E. Activity-dependent changes in the Nedd4-1 interactome

In Chapter IV, we further explored Nedd4-1's function in neurons by using APEX2 mediated proximity labeling to characterize Nedd4-1's interactome. I created and validated the utility of APEX2-HA-Nedd4-1 expression and proximity labeling in primary neuronal cultures. Strikingly, we found that Nedd4-1's interactome changes in an activity-dependent manner. We show that in untreated neuronal cultures, APEX2-Nedd4-1 had significantly

enriched association with spliceosome complex components and proteins involved in mRNA processing. In contrast, NMDAR activation significantly increased APEX2-Nedd4-1 interaction with synapse associated proteins including synaptic organization proteins. These findings are supported by distinct activity-dependent trafficking dynamics of GFP-Nedd4-1 in dendrites and at synapses described in Chapter III, including data which show that Nedd4-1's recruitment to synapses, defined by co-localization with the postsynaptic density marker protein PSD-95, is significantly increased in response to NMDAR activation. We confirm the functional relevance of NMDAR regulation of Nedd4-1 in neurons through the first use of 1-benzyl-I3C in neurons, an inhibitor of Nedd4-1 activity, to show that NMDAR-dependent regulation of GluA1-containing AMPARs directly involves Nedd4-1.

E. References

Huganir RL, Nicoll RA. AMPARs and synaptic plasticity: the last 25 years. Neuron. 2013;80(3):704-717. doi:10.1016/j.neuron.2013.10.025

Turrigiano GG. The self-tuning neuron: synaptic scaling of excitatory synapses. Cell. 2008;135(3):422-435. doi:10.1016/j.cell.2008.10.008

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function [published correction appears in Pharmacol Rev. 2014 Oct;66(4):1141]. Pharmacol Rev. 2010;62(3):405-496. doi:10.1124/pr.109.002451

Paoletti P, Bellone C, Zhou Q. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat Rev Neurosci. 2013;14(6):383-400. doi:10.1038/nrn3504

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function [published correction appears in Pharmacol Rev. 2014 Oct;66(4):1141]. Pharmacol Rev. 2010;62(3):405-496. doi:10.1124/pr.109.002451

Cull-Candy SG, Leszkiewicz DN. Role of distinct NMDA receptor subtypes at central synapses. Sci STKE. 2004;2004(255):re16. Published 2004 Oct 19. doi:10.1126/stke.2552004re16

Paoletti P. Molecular basis of NMDA receptor functional diversity. Eur J Neurosci. 2011;33(8):1351-1365. doi:10.1111/j.1460-9568.2011.07628.x

Sakimura K, Kutsuwada T, Ito I, Manabe T, Takayama C, Kushiya E, Yagi T, Aizawa S, Inoue Y, Sugiyama H, Mishina M. Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. Nature. 1995;373(6510):151-155. doi:10.1038/373151a0

Brigman JL, Wright T, Talani G, Prasad-Mulcare S, Jinde S, Seabold GK, Mathur P, Davis MI, Bock R, Gustin RM, Colbran RJ, Alvarez VA, Nakazawa K, Delpire E, Lovinger DM, Holmes A. Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci. 2010;30(13):4590-4600. doi:10.1523/JNEUROSCI.0640-10.2010

Hardingham G. NMDA receptor C-terminal signaling in development, plasticity, and disease. F1000Res. 2019;8:F1000 Faculty Rev-1547. Published 2019 Aug 30. doi:10.12688/f1000research.19925.1

Man HY. GluA2-lacking, calcium-permeable AMPA receptors--inducers of plasticity?. Curr Opin Neurobiol. 2011;21(2):291-298. doi:10.1016/j.conb.2011.01.001

Diering, G. H., & Huganir, R. L. (2018). The AMPA Receptor Code of Synaptic Plasticity. Neuron, 100(2), 314–329. https://doi.org/10.1016/j.neuron.2018.10.018

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Hipp MS, Kasturi P, Hartl FU. The proteostasis network and its decline in ageing. Nat Rev Mol Cell Biol. 2019;20(7):421-435. doi:10.1038/s41580-019-0101-y

Malgaroli A, Vallar L, Zimarino V. Protein homeostasis in neurons and its pathological alterations. Curr Opin Neurobiol. 2006;16(3):270-274. doi:10.1016/j.conb.2006.05.009

Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem. 2001;70:503-533. doi:10.1146/annurev.biochem.70.1.503

Akutsu M, Dikic I, Bremm A. Ubiquitin chain diversity at a glance. J Cell Sci. 2016;129(5):875-880. doi:10.1242/jcs.183954

Swatek KN, Komander D. Ubiquitin modifications. Cell Res. 2016;26(4):399-422. doi:10.1038/cr.2016.39

Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron. 2003;40(3):595-607. doi:10.1016/s0896-6273(03)00687-1

Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron. 2003;40(3):595-607. doi:10.1016/s0896-6273(03)00687-1

Bingol B, Schuman EM. A proteasome-sensitive connection between PSD-95 and GluR1 endocytosis. Neuropharmacology. 2004;47(5):755-763. doi:10.1016/j.neuropharm.2004.07.028

Guo L, Wang Y. Glutamate stimulates glutamate receptor interacting protein 1 degradation by ubiquitin-proteasome system to regulate surface expression of GluR2. Neuroscience. 2007;145(1):100-109. doi:10.1016/j.neuroscience.2006.11.042

Emtage L, Chang H, Tiver R, Rongo C. MAGI-1 modulates AMPA receptor synaptic localization and behavioral plasticity in response to prior experience. PLoS One. 2009;4(2):e4613. doi:10.1371/journal.pone.0004613

Patrick GN, Bingol B, Weld HA, Schuman EM. Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. Curr Biol. 2003;13(23):2073-2081. doi:10.1016/j.cub.2003.10.028

Schaefer H, Rongo C. KEL-8 is a substrate receptor for CUL3-dependent ubiquitin ligase that regulates synaptic glutamate receptor turnover. Mol Biol Cell. 2006;17(3):1250-1260. doi:10.1091/mbc.e05-08-0794

Cortese GP, Zhu M, Williams D, Heath S, Waites CL. Parkin Deficiency Reduces Hippocampal Glutamatergic Neurotransmission by Impairing AMPA Receptor Endocytosis. J Neurosci. 2016;36(48):12243-12258. doi:10.1523/JNEUROSCI.1473-16.2016

Kim JE, Lee DS, Kim MJ, Kang TC. PLPP/CIN-mediated NEDD4-2 S448 dephosphorylation regulates neuronal excitability via GluA1 ubiquitination. Cell Death Dis. 2019;10(8):545. Published 2019 Jul 18. doi:10.1038/s41419-019-1781-0

Ghilarducci K, Cabana VC, Desroches C, Chabi K, Bourgault S, Cappadocia L, Lussier MP. Functional interaction of ubiquitin ligase RNF167 with UBE2D1 and UBE2N promotes ubiquitination of AMPA receptor. FEBS J. 2021;288(16):4849-4868. doi:10.1111/febs.15796

Ma P, Wan LP, Li Y, He CH, Song NN, Zhao S, Wang H, Ding YQ, Mao B, Sheng N. RNF220 is an E3 ubiquitin ligase for AMPA receptors to regulate synaptic transmission. Sci Adv. 2022;8(39):eabq4736. doi:10.1126/sciadv.abq4736

Pavlopoulos E, Trifilieff P, Chevaleyre V, Fioriti L, Zairis S, Pagano A, Malleret G, Kandel ER. Neuralized1 activates CPEB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage. Cell. 2011;147(6):1369-1383. doi:10.1016/j.cell.2011.09.056

Huo Y, Khatri N, Hou Q, Gilbert J, Wang G, Man HY. The deubiquitinating enzyme USP46 regulates AMPA receptor ubiquitination and trafficking [published correction appears in J Neurochem. 2017 May;141(3):472]. J Neurochem. 2015;134(6):1067-1080. doi:10.1111/jnc.13194

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Widagdo J, Chai YJ, Ridder MC, Chau YQ, Johnson RC, Sah P, Huganir RL, Anggono V. Activity-Dependent Ubiquitination of GluA1 and GluA2 Regulates AMPA Receptor Intracellular Sorting and Degradation. Cell Rep. 2015;10(5):783-795. doi:10.1016/j.celrep.2015.01.015

Widagdo J, Kerk JW, Guntupalli S, Huganir RL, Anggono V. Subunit-Specific Augmentation of AMPA Receptor Ubiquitination by Phorbol Ester. Cell Mol Neurobiol. 2020;40(7):1213-1222. doi:10.1007/s10571-020-00809-2

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Azarnia Tehran D, Kochlamazashvili G, Pampaloni NP, Sposini S, Shergill JK, Lehmann M, Pashkova N, Schmidt C, Löwe D, Napieczynska H, Heuser A, Plested AJR, Perrais D, Piper RC, Haucke V, Maritzen T. Selective endocytosis of Ca2+-permeable AMPARs by the Alzheimer's disease risk factor CALM bidirectionally controls synaptic plasticity. Sci Adv. 2022;8(21):eabl5032. doi:10.1126/sciadv.abl5032

Zhu M, Cortese GP, Waites CL. Parkinson's disease-linked Parkin mutations impair glutamatergic signaling in hippocampal neurons. BMC Biol. 2018;16(1):100. Published 2018 Sep 10. doi:10.1186/s12915-018-0567-7

Cortese GP, Zhu M, Williams D, Heath S, Waites CL. Parkin Deficiency Reduces Hippocampal Glutamatergic Neurotransmission by Impairing AMPA Receptor Endocytosis. J Neurosci. 2016;36(48):12243-12258. doi:10.1523/JNEUROSCI.1473-16.2016

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Lin A, Hou Q, Jarzylo L, Amato S, Gilbert J, Shang F, Man HY. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. J Neurochem. 2011;119(1):27-39. doi:10.1111/j.1471-4159.2011.07221.x

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Hou Q, Gilbert J, Man HY. Homeostatic regulation of AMPA receptor trafficking and degradation by light-controlled single-synaptic activation. Neuron. 2011;72(5):806-818. doi:10.1016/j.neuron.2011.10.011

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Chapter II

A review of Nedd4-1’s role in the CNS in health and disease

A. Abstract

Protein homeostasis (proteostasis) is crucial to the health of neurons and the continued functioning of the brain. The ubiquitin-proteasome system (UPS), which is one of the major degradation pathways in eukaryotic cells, plays an important role in maintaining proteostasis in the neuron. In this review, I will highlight the ubiquitin E3 ligase, neural precursor cell expressed developmentally down-regulated protein 4-1 (Nedd4-1)'s, importance in healthy function of the central nervous system (CNS). I will summarize Nedd4-1's role in neurite growth, ionotropic receptor trafficking, Oxygen homeostasis, and many other biological pathways. I will also detail Nedd4-1 as a possible mechanism in neuronal degeneration, psychiatric disease, glioma, and other neurological disorders. In total, this review will eloquently present the rising importance of Nedd4-1 in understanding brain function.

B. Introduction

There are tens of thousands of unique proteins expressed at any given moment that all contribute to the normal and healthy function of the cell. Each of these proteins exist in varying abundances. Protein homeostasis is the regulation of protein abundance to maintain normal and healthy cellular function. This is important throughout the body and especially in the central nervous system (CNS) due to the dynamic and ever adaptive nature of neurons. Protein homeostasis can be broken into three parts: synthesis, maintenance, and degradation. For a long time, the study of disruptions in protein homeostasis were focused in dysregulated

protein synthesis, but as the field developed, protein degradation has become just as important of a study.

One of the key players in protein degradation is the ubiquitin proteasome system (UPS). Ubiquitin is a small molecule that can bind to lysine residues on substrates or other ubiquitin molecules to target these proteins to the proteasome/lysosome for degradation or many other molecular pathways in the cell. Ubiquitination of proteins is mediated by three ubiquitin interacting enzymes (E1 activating enzyme, E2 conjugating enzyme, and E3 ligating enzyme). These enzymes work together to activate ubiquitin molecules and target them to specific substrates based on the binding affinity of the E2 and/or E3 enzymes. There are hundreds of E3 ligases described in the literature, but this review will be focusing on the relevance of Nedd4-1. Extensive studies have been done on Nedd4-1 detailing its importance in numerous biological pathways. Notably a huge branch of literature is dedicated to uncovering Nedd4-1's role in regulating tumorigenesis and tumor progression in many types of cancers. This review particularly seeks to highlight Nedd4-1's well documented role in the CNS. Nedd4-1 has been implicated as a mechanism for various biological processes necessary for neuronal function and as having a role in the pathophysiology of several neurological disorders. The more we understand about this ligase and its function, the better understanding we will have of protein degradation's role in maintaining healthy brain function.

Nedd4-1 History

Nedd4 was discovered in 1992. Kumar et al 1992, used subtraction cloning to identify family of cDNA clones, NEDD[1-10], that were downregulated during mouse brain

development (Kumar S. et al, 1992). They followed these experiments up by cloning the Nedd4 cDNA specifically to understand its structure and function. They described a calcium binding (C2) domain, three putative protein–protein interaction (WW) domains, and a C-terminus region similar to the ubiquitin–protein ligase (HECT) domain. They find that Nedd4 is expressed in the CNS in neurogenesis and less over development (Kumar S. et al, 1997; Anan T. et al, 1998). Subcellular localization experiments reveal Nedd4 is found in cytoplasm and cytoplasmic periphery unless the C2 domain is deleted and then Nedd4 localizes more to the nuclear and perinuclear region. Further studies found that the C2 domain is specifically responsible for calcium dependent localization of Nedd4 to the plasma membrane localization and apical regions of the cell (Plant P. et al, 1997; Plant P. et al, 2000).

The first exploration of Nedd4-1 function came from a 1996 in the study of a rare genetic disorder called Liddle's Syndrome. This disease is hallmarked by abnormal kidney function that leads to inheritable hypertension. What was known at the time was that people with Liddle's syndrome show a mutation in their epithelial sodium (Na+) channels (ENaCs) that deletes the second of two (P1 and P2) proline rich regions. Staub et al 1996, was able to uncover that the WW protein binding domains of Nedd4 bind to proline rich regions (xPPxY domains) like the ones mutated in these ENaCs. Without this region Nedd4 was not able to degrade these ENaCs leading to a build-up in in the kidneys and an increase in salt uptake (Stuab O. et al, 1997; Goulet C. et al, 1998; Kanelis V. et al, 1998; Abriel H. et al, 1999; Farr T. et al, 2000). Studies show Nedd4 expression in the same region as epithelial channels in multiple parts of the body (Staub O. et al, 1997), as well as ubiquitination of ENaCs by Nedd4 decreasing the channel half-life (Staub O. et al, 1997; Harvey K. et al, 1999).

Following this uncovering of Nedd4-1 function in Liddle's Syndrome, many labs explored further functionality of Nedd4. Several of these studies defined Nedd4 interactions with novel substrates (Joliffee C. et al, 2000). One study showed that Nedd4 may down regulate epithelial Na+ channels in the salivary duct in an Intracellular Na+ dependent manner involving the Go pathway (Dinudom A. et al, 1998). Others showed Nedd4 regulates cardiac voltage gated Na+ channels (Abriel H. et al, 2000) and associates with adaptor protein Grb10 which is known to interact with insulin and insulin like growth factor receptors (Morrione A. et al, 1999). Other studies at this time also proposed regulatory mechanisms for Nedd4. Harvey K. et al 1998, described an apoptotic regulation of Nedd4, claiming that the protein is cleaved by a caspase during apoptosis (Harvey K. et al, 1998). A number of studies implicate a role for Nedd4 in viral infection. They suggest that Nedd4 regulates the latent membrane protein 2A (LMP2A) which in turn regulates B-cell activity after infection with Epstein-Barr virus (EBV) (Ikeda M. et al, 2000; Winberg G. et al, 2000). Nedd4 also seems to play a role in retroviral release (Strack B. et al, 2000). The Vp40 protein of the Ebola virus contains a PPxY motif that is recognized by Nedd4's WW domain (Harty RN. et al, 2000).

It wasn't until 2001 that the field realized Nedd4 represented more than one distinct protein. The Staub lab discovered Nedd4-2 or Nedd4-like (Nedd4L) protein and isolated it from Nedd4-1 (Kamynina E. et al, 2001). Three years later, the Kumar lab for the first time since their earlier characterization and localization experiments, described a role for Nedd4-1 in the central nervous system (Fotia AB. et al, 2004). From here the field exploded as people began to realize how critical of a role Nedd4-1 plays in CNS function in health and disease.

Nedd4-1 Structure

Much of the insight on Nedd4-1's function arose from an understanding of the E3 ligase's structure. Nedd4 is made up of nine family members in humans: Nedd4-1 (also known as NEDD4), Nedd4-2 (NEDD4L), Itch, WW domain-containing E3 ubiquitin protein ligase 1 (WWP1), WWP2, NEDL1 (HECW1), NEDL2 (HECW2), SMAD-specific E3 ubiquitin protein ligases (Smurf1) and Smurf2 (Scheffner M. et al, 2014; Huang X. et al, 2019). Nedd4 proteins consist of three distinct domains that dictate different functional aspects of the ligase. At the N-terminus is the calcium binding (C2) domain. This domain is conserved and found in many other proteins and mediates localization to the plasma membrane through phospholipid binding in a Ca^{2+} dependent manner (Dunn R. et al, 2004). These ligases also have anywhere from two to four protein-protein interactions domains (WW domains) of about 40 amino acids in length that contain two conserved tryptophan (W) residues (Boase NA. et al, 2015). The WW domain binds proline rich PY (PPxY) motifs and serine/threonine residues on its substrates (Sudol M. et al, 1995). Differences in the number and binding affinity of the WW domain accounts for much of the variation in function between Nedd4 family members (Dodson EJ. et al, 2015). At the C-terminus of the protein is the catalytic domain. This domain was found to be similar to the one in human E6-AP (the papilloma virus oncoprotein E6-associated protein), the first discovered ubiquitin-protein ligase. This HECT (Homologous to the E6-AP C-terminus) domain contains an N-terminus lobe meant for binding with E2 conjugating enzymes as well as a C-terminus lobe that contains a conserved cysteine residue that forms a thioester bond with activated ubiquitin, before catalysing the ubiquitination of a lysine in the substrate protein (Rotin D. et al, 2009; Boase NA. et al, 2015). In addition to Nedd4-1 domains' binding affinities with other

molecules, there are interactions between the domains themselves. The HECT domain holds an affinity for the C2 domain in some Nedd4 proteins and certain WW domains in others that allow for an autoinhibitory regulation of the HECT ligase activity. This autoinhibition can then be released by protein and/or Ca^{2+} binding (Chen C. et al, 2007; Zhou W. et al, 2014). Such thorough knowledge of Nedd4-1's structure has guided our understanding of this proteins function throughout the body and particularly in the CNS.

C. Nedd4-1 Function in Health

I. Nedd4-1 Knockout Mice

For a long time, scientists have learned about the function of a protein, brain circuit or even region through ablation or removal. A lot has been discovered about the function of Nedd4-1 in the brain using Nedd4-1 Knockout (KO) animal models. These models have provided insight on Nedd4-1's role in successful formation of neuromuscular junctions, movement, as well as regulation of long-term potentiation (LTP) in the central nervous system (CNS). In one of the earlier studies using Nedd4-1 KO models, Lui et al. used this model to show that a complete deletion of Nedd4-1 seems to affect the healthy formation of neuromuscular junctions and lead to perinatal lethality (Liu Y. et al, 2009). Also, Nedd4-1 seems to be playing an important role in healthy cell growth and development throughout the entire CNS seeing as Nedd4-/- mice show an overall reduction in cell number and size. This included a reduction in size the of muscle fibers and the volume of dorsal-root ganglion (DRG) neurons in the spinal cord. A closer look shows that Nedd4-/- is not involved in motor axon targeting but is important to facilitating nerve-muscle interaction. Lui et al. suggest that

the mechanism by which Nedd4-1 helps regulate nerve fasciculation is likely non-cell autonomous due to the fact that they found Nedd4-1 expression in skeletal muscle and Schwan cells but not the motor neurons themselves. Camera et al. followed these studies up using a heterozygous Nedd4-1 KO model to study the behavior of adult mice and describe a characteristically abnormal gait present in these mutant mice (Camera D. et al, 2014). Six-month-old Nedd4+/- mice show increases in stride length, stride duration, propulsion duration, braking duration, and swing of motion. Although the cerebellum plays a huge role in motor coordination, this study did not find any histological evidence of degeneration of Purkinje cells. Camera et al. also observed redistribution GluR1 into large punctate structures in Nedd4+/- mice and cite disruption of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activity as a possible mechanism for the abnormal gait in these mutant mice. The same lab published a study almost two years later claiming that not only are there motor deficits in Nedd4+/- mice but a marked effect on cognitive function and synaptic plasticity (Camera D. et al, 2016). Using the Morris water maze, this study shows that Nedd4+/- mice have decreased long-term spatial learning and memory. In contrast, they did not observe the same deficit in Short-term spatial memory in shown using the Y-maze test. Camera et al suggest that this deficit may be caused by alterations in the post synaptic membrane caused by the 50% decrease in Nedd4-1 in KO mice. Wherein using paired pulse facilitation (PPF) showed no change in the integrity of pre-synaptic terminals, PPF and Immunohistochemistry showed decreased integrity of post-synaptic terminals and a decrease of Nedd4-1 protein levels in the CA1 region, a region responsible for spatial learning and memory. Together these studies begin to suggest role for Nedd4-1 in the CNS as a whole and

define a Nedd4-1 KO model that can be used to further the understanding of Nedd4-1 function.

II. Axon Branching

Axon branching is an import process in neuronal development where the axonal projection of the neuron branches to form new synapses with the surrounding neurons. This process is crucial to establishing healthy neural networks. It is well known that Nedd4-1 plays a role in axon outgrowth and branching, but the exact mechanisms of Nedd4-1's involvement is still unknown and somewhat debated in the field. Kawabe et al. was one of the earlier studies to describe Nedd4-1's involvement in axon branching and suggest a mechanism of action (Kawabe H. et al, 2010). Studying Nedd4-1 KO cultured neurons, this study was able to show that these cells exhibited shorter and less complex dendrites, a phenotype that was rescued by re-expression of Nedd4-1 in these KO cultures. Using biochemical techniques, the lab shows Nedd4-1 regulating axonal growth by forming a binding complex with serine/threonine kinases, TNIK and Rap2A, where TNIK serves as an adaptor allowing Nedd4-1 to ubiquitinate Rap2A. Both TNIK and Rap2A are known modulators of the cytoskeleton and cell morphology (Fu Z. et al, 2007; Taira K. et al, 2004). With further experimentation they were able to show that ubiquitination of Rap2A disrupted its ability to interact with other proteins. The upregulation in Rap2A activity in Nedd4-1 KO cultures suggests that Nedd4-1 is likely the mediator of that ubiquitination (Kawabe H. et al, 2010). Another study, (Christie KJ. et al, 2012), suggests another mechanism of Nedd4-1 regulation of axonal branching and outgrowth. Here they look at Nedd4-1's role in neurite outgrowth

after injury in the peripheral nervous system, specifically in DRG neurons, satellite cells, and the sciatic nerve. In this study, they not only show a role for UPS in axon growth and maintenance, but also show co-expression of PTEN and Nedd4-1 in the regions of interest. PTEN is well known in the literature for its role in regulating the PI3K pathway and limiting cell proliferation. There have been conflicting studies in the literature describing the relationship between PTEN and Nedd4-1. This lab claims to show evidence of Nedd4-1 playing a regulatory role over PTEN. They show that when you knock out Nedd4-1 you get a decrease in neurite outgrowth that can be rescued by inhibiting PTEN itself. This PTEN inhibition leads to greatly increased neurite outgrowth suggesting that Nedd4-1 ubiquitination and reduction of PTEN allows for axonal growth. But there are conflicting reports on the interaction between Nedd4 and PTEN. (Hsia HE. et al, 2014) suggests the reverse relationship between PTEN and Nedd4-1. Their studies claim to show that a very small percentage of endogenous PTEN in the CNS is ubiquitinated in the developing mouse brain. They suggest that PTEN inhibits the PI3K-mTORC1 pathway to limit Nedd4-1 mRNA translation and neurite growth. They show evidence not only that Nedd4-1 protein levels are increased in PTEN KO cells, but they also show an increase in Nedd4-1 mRNA association with translational bodies. This supports the theory that PTEN and mTORC1 pathways lie upstream of Nedd4-1 regulating its translation and ability to promote neurite growth.

III. Sexual Differentiation

Some believe that sexual differentiation of the mammalian brain happens through complex interplay of diverse hormonally regulated mechanisms, including cell survival and

death, neurogenesis, cell morphological differentiation, as well as epigenetic control of gene expression. Nedd4-1 has been implicated in the sexual differentiation of the developing mouse brain. Shakakibara et al. used microarray analysis on male and female mice brains to isolate and describe 12 genes, known to have a role in neuronal development, that showed significant differences in expression between the genders (Shakakibara M. et al, 2013). Nedd4 happened to be one of these genes that showed decreased expression in the hypothalamus of male mice. In their studies they also find that WT mice pretreated with estrogen show a decrease in Nedd4 expression. Their lab suggests a couple mechanisms by which Nedd4 and the other indicated genes may be playing a role in sexual differentiation.

IV. Receptor Regulation

AMPA Receptors

AMPA receptors (AMPARs) mediate the large majority of fast-acting excitatory synaptic transmission in the CNS (Shepard JD. et al, 2007). Understanding AMPARs is fundamental to understanding synapse and circuit plasticity and the mechanism of learning and memory in the brain. The first study to implicate Nedd4-1's role in regulating AMPARs in the CNS came out of our lab. Schwarz et al. showed that Nedd4-1 mediates ubiquitin dependent turnover of AMPARs (Schwarz LA. et al, 2010). Using Immunohistochemistry, receptor point mutations, and biochemical techniques the lab was able to show that AMPA-mediated ubiquitination of the GluA1 subunit of the AMPA receptor is crucial to the degradation of these receptors. This was followed up with RNAi conditional knockdown of Nedd4-1 which prevented the ubiquitination and degradation of GluA1, suggesting Nedd4-1's

role in AMPAR ubiquitination and turnover. Another study published around the same time repeat and corroborate these findings. Lin et al. similarly finds that the ubiquitination of the GluA1 subunit of the AMPA receptor is crucial to its internalization mediated by Nedd4-1 (Lin A. et al, 2011). What they do not observe in this study is the activity-dependent ubiquitination of AMPARs described in Schwarz et al. This lab published another study the same year, Hou et al., observing that light stimulation at a single synapse may drive AMPA receptor internalization and Nedd4-1 gathering at the synapse (Hou Q. et al, 2011). Using light-gated glutamate receptors in combination with immunohistochemical techniques, this study was able to show a decrease in GluA1 abundance specifically at stimulated sites. This decrease was blocked by silencing synaptic activity (TTX), a removal of calcium, inhibiting N-methyl D-aspartate (NMDA) receptors (APV), protease inhibitor (MG132), and AMPA receptor inhibitor CNQX but not the more selective AMPA receptor inhibitor (GYKI). All of these may play a role in regulating AMPA receptor turnover and are likely mediated through Nedd4-1 activity. Their study showed that single synapse light activation increased the abundance of Nedd4-1 at stimulated sites where you see this decrease in AMPAR abundance. This idea emerging in the field of understanding AMPA and NMDA receptor's role in Nedd4-1 mediated AMPA receptor turnover is supported even more by another study out of our lab, (Scudder SL. et al, 2014). In this study, the lab shows that AMPA receptor activation drives Nedd4-1 to become punctate and co-localize with the synapse. This recruitment of Nedd4-1 to the synapse is shown to be AMPAR-dependent and NMDAR-independent as well as calcium mediated. The calcium-dependent element to this phenotype lies in line with Nedd4-1 structure with a calcium binding domain responsible for subcellular localization of the ligase.

Redistribution of Nedd4-1 was observed within 2min of activation of the synapse and persisted through 20min. Although both AMPA and Glutamate/Glycine activation drove Nedd4-1 recruitment to the synapse, Glutamate/Glycine did not drive AMPAR ubiquitination until you introduce NMDA receptor inhibitor APV. The lab suggests a possible mechanism for NMDARs to counteract Nedd4-1 recruitment and ubiquitination of AMPARs through the deubiquitinating enzyme USP8. Wei et al. propose another possible mechanism of Nedd4-1 mediated AMPAR regulation through histone modification of the Nedd4 gene (Wei J. et al, 2016). This lab shows that in stressed animals there is an increased expression of histone deacetylase 2 (HDAC2) which may lead to a loss of repressive histone methylation at the Nedd4 promoter and increased expression of Nedd4-1. This leads downstream to an increase in GluA1 ubiquitination of and loss of functional AMPA receptors.

Other Receptors

Nedd4-1 has also been implicated in the regulation of other receptors in the brain besides the AMPA receptor. Gautam et al. suggests that Nedd4-1 may play a part in the regulation of the GluN2D subunit of the NMDA receptor (Gautam V. et al, 2013). They used proteomic and yest two-hybrid studies to show that Nedd4-1 WW substrate binding domains do in fact interact with the PPxY binding site on the GluN2D subunit. They were also able show that overexpression of active Nedd4-1 decreased the amount of functional GluN1/GluN2D receptor expression in Xenopus oocytes. Lee et al. claims a role for Nedd4-1 in the regulation of metabotropic glutamate receptor 7 (mGluR7) (Lee S. et al, 2019). They observe that β-arrestin forms a complex with Ned4-1 and mediates mGluR7 ubiquitination in an activity-dependent manner. Biochemical analysis is used to show β-arrestin and Nedd4-1

binding to mGluR7 in an activity-dependent manner as well as regulation of mGluR7 ubiquitination and endocytosis. Outside of glutamatergic receptors, Nedd4-1 has been associated with the regulation of Fibroblast growth factor receptor 1 (FGFR1) as well. Persaud et al. makes the claim that Nedd4-1 ubiquitinates and downregulates FGFR1 at the cell surface, and uses biochemical techniques describe a novel binding site outside of the recognized PY motif were Nedd4-1 may be binding these receptors (Persaud A. et al, 2011). In this study, the lab also suggests that through Nedd4-1's regulation of FGFR1, the ligase shows an enhancing effect on neuronal differentiation of human embryonic neural stem cells.

V. Phosphorylation

Much is still unknown about the post-translational modification of Nedd4-1 in the CNS. Persaud et al. is one of the few studies to explore this topic (Persaud A. et al, 2014). In this study, they show that the phosphorylation state of Nedd4-1 may play a huge role in regulating its activity. Their lab was able to show that activation of FGFR1 increased the tyrosine phosphorylation of Nedd4-1. They then used Nedd4-1 mutants and biochemical techniques to show that when specific sites in the C2-domain and HECT domain are phosphorylated, Nedd4-1 shows an increase in activity via autoubiquitination. The phosphorylation of these sites seems to disrupt the autoinhibitory interaction between the C2 and HECT domains. The lab also suggests that this phosphorylation of Nedd4-1 is mediated through c-src kinase activity. These findings are a good start to understanding the mechanics of how Nedd4-1 is regulated in the neuron.

VI. Gap Junction Regulation

Gap Junctions play an important role in communication between cells in the brain. A gap junction channel is generated by the docking of two end-to-end hemichannels, termed connexons, in the opposing plasma membranes (Giaume C. et al, 2010). Astroglial networks are commonly interconnected through these gap junctions which allow diffusion of second messengers, ions, and small metabolites between adjacent astrocytes (Giaume C. et al, 2010). Liao et al. shows evidence that astrocytes treated with an endotoxin, lipopolysaccharide (LPS), show a decrease in connexin43 (Cx43) expression mediated through Nedd4-1 activity (Liao CK. et al, 2013). Treatment with LPS not only showed a decrease in Cx43 levels and an increase in ubiquitination of Cx43, but also increase in Nedd4-1 binding with Cx43. This study also suggests that a stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) mediates Nedd4-1 and Cx43's interaction by showing a decrease in the ubiquitination of Cx43 and its interaction with Nedd4-1 with JNK inhibitors.

VII. Oxygen Homeostasis

Molecular oxygen is incredibly important to a cell's ability to function through its involvement in ATP production and many cell signaling pathways. This is even more important in the central nervous system due to the brain's proportionally high energy demands. Oxygen deprivation, hypoxia, leads to energy crisis and possibly cell death. Therefore, it is essential for a cell to be able to monitor and respond to a hypoxic state. Recent studies show a possibility that astrocytes are capable of sensing a wide variety of environmental factors, such as CO2 and O2. Uchiyama et al. reveals a role for TRPA1 cation

channels to mediate the selective sensing of moderate hypoxia (Uchiyama M. et al, 2020). This study suggests that hypoxia diminishes Nedd4-1 ubiquitination and internalization of TRPA1 channels. Without this interaction, TRPA1 channels can accumulate at the plasma membrane and increase Ca^{2+} influx, accelerate ATP release, and increase amplitude of inspiratory discharge rhythm.

VIII. Drug Mechanism

For many drugs that are known to have an effect in the brain or in neurological disorders, there is very little known about the mechanisms by which these drugs have an effect. A couple of studies in the literature have used proteomic and bioinformatics to link Nedd4-1 to several drugs' mechanisms of action. Duncan et al. suggests that Nedd4-1 is playing a role in the mechanism of some antipsychotic drugs commonly used to treat the psychotic symptoms of Schizophrenia (Duncan CE. et al, 2008). NEDD4 was amongst the genes to show a significant difference between untreated and Olanzapine treated brain lysates. Olanzapine treated brain lysates also showed a marked decrease in actual Nedd4-1 protein levels. This study suggests that Nedd4-1's effect on Olanzapine's mechanism of action could be through regulating voltage-gated channels. Another study, (Stockton SD Jr. et al, 2015) uses mass spectrometry and analysis in untreated and morphine treated brains. They report NEDD4 as one of the significant intermediates in morphine induced changes at striatal PSDs; however, very little is known about the role of Nedd4-1 at the synapse during addiction to morphine.

D. Nedd4-1 Function in Disease

I. Dementia

Neurodegenerative diseases are characterized by the progressive loss of select populations of neurons and the presentation of motor, cognitive and behavioral deficits. Some of the most common of these disorders include Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (AD), and amyotrophic lateral sclerosis (ALS) (Dugger B. et al, 2017). The mechanism of these disorders is not fully understood, but disruptions in the ubiquitin/proteasome system and autophagosomal/lysosomal systems seem to be present among many of these disorders. It is well documented in the literature that Nedd4-1, specifically, plays a critical role in many neurodegenerative diseases' pathogenesis. Kwak et al. shows Nedd4-1 regulating insulin growth factor 1 receptor (IGF-1R) – a tyrosine kinase with a well-known role in numerous intracellular signaling cascades and presence in the pathophysiology of more than one neurodegenerative disease – in a excitotoxic zinc or ROS induced model of neurodegeneration (Kwak Y. et al, 2012). They also show that NEDD4-1 expression is upregulated in postmortem human AD, PD, and HD brains and in the spinal cords of ALS cases. This section focuses on detailing Nedd4-1's described role AD and PD.

Alzheimer's Disease

Alzheimer's Disease (AD) is a well-studied cause of dementia and damage in the CNS. This disease is characterized by neurodegeneration, neuronal loss, and atrophy (Oboudiyat C. et al, 2013). Some of the well-known pathological hallmarks of AD are toxic

amyloid-beta (Aβ) oligomers and neurofibrillary tangles. Nedd4-1 has also been shown to play a significant role in the pathophysiology of AD. The prevailing theory in the literature is that Nedd4-1 mediates Aβ induced synaptic dysfunction through the regulation of AMPA receptor turnover. Many studies have implicated Nedd4-1 in the regulation of AMPARs by Aβ. one of the first from our lab, show that Aβ treatment in neurons causes a decreased mEPSC frequency and amplitude (Rodrigues EM. et al, 2016), spine density (Rodrigues EM. et al, 2016), surface levels of GluA1 (Rodrigues EM. et al, 2016; Guntupalli S. et al, 2017; Zhang Y. et al, 2018), and ubiquitination of GluA1 (Guntupalli S. et al, 2017; Zhang Y. et al, 2018) and GluA2/3 (Rodrigues EM. et al, 2016) in neurons. Ubiquitination is necessary for the Aβ mediated reduction in surface GluA1 (Guntupalli S. et al, 2017) and half-life of these receptor subunits (Zhang Y. et al, 2018). Aβ treatment was also shown to increase total Nedd4-1 levels (Akkaya BG. et al, 2015; Zhang Y. et al, 2018) and drive Nedd4-1 recruitment to the synapse (Rodrigues EM. et al, 2016). Inhibiting Nedd4-1 blocks Aβ induced deficits in mEPSC amplitude, spine density, and surface GluA1 levels (Rodrigues EM. et al, 2016; Zhang Y. et al, 2018). Guntupalli et al. even suggests a possible mechanism of interplay between Nedd4-1 and S845 phosphorylation on GluA1 (Guntupalli S. et al, 2017). In this study they show that phosphomimetic S845 blocks ubiquitination of GluA1, Nedd4-1 binding to GluA1, and Aβ induced reduction in surface levels of GluA1. They suggest that S845 phosphorylation plays an antagonistic role to GluA1 ubiquitination (Guntupalli S. et al, 2017). Other labs have suggested alternative roles for Nedd4-1 in AD pathophysiology. Multiple studies suggest a mechanism of increased toxic Aβ oligomers through they dysregulation of the ATP Binding Cassette transporter ABCB1 by Nedd4-1 (Akkaya BG. et al, 2015; Chai

AB. et al, 2022). Akkaya et al. describes an increase in Nedd4-1 levels and a decrease in ABCB1 levels in Aβ treated cells (Akkaya BG. et al, 2015). Another study, (Correani V. et al, 2019), proposes a mechanism of Aβ's effect on Nedd4-1. They observe that in Aβ treated microglia, there is an increase in PARylated Nedd4-1 indicating a possible mechanism of Nedd4-1 regulation.

Parkinson's Disease

One of the fastest growing causes of disability from neurological disorders is Parkinson's disease (PD) (Dorsey ER. et al, 2018). PD was described in 1817 by James Parkinson and is characterized by rest tremor, bradykinesia, rigidity and postural instability, and a variety of other motor and non-motor symptoms (Jankovic J. et al, 2020). In this disease, brain cells accumulate aggregates of altered α-synuclein (aS) that are thought to underlie cellular toxicity and hence disease (Geodert M. et al, 2013). Throughout the literature, Nedd4-1 has been shown to play an important role in the regulation of aS or the mechanism of aS toxicity. Many studies in the field implicate Nedd4-1 in the ubiquitination of aS (Tofaris GK. et al, 2011; Sugeno N. et al, 2014; Mund T. et el, 2018; Won SY. et al, 2022) and downregulation of aS protein (Tofaris GK. et al, 2011; Davies SE. et al, 2014), but there are differing claims on what forms of aS that Nedd4-1 binds and interacts with. While (Sugeno N. et al, 2014), states that Nedd4-1 interacts more with aS internalized from the extracellular space than de novo aS already in the cell, (Mund T. et al, 2018), suggests that Nedd4-1 interacts with and ubiquitinates wild-type (WT) aS filaments significantly more than the toxic A53T mutant aS. This lab goes so far as to suggest that this may be a mechanism of mutant aS effectiveness in driving disease if it cannot be downregulated by Nedd4-1. Recent studies have even tried to

utilize this interaction to create “ubiquibodies”, Nedd4-1 construct fused to aS scFv, to target Nedd4-1 directly to aS as a possible mechanism of aS downregulation (Vogiatzis S. et al, 2021). Although a majority of the studies on this topic indicate a role for Nedd4-1 upstream of aS, (Kim E. et al, 2016) implicate Nedd4-1 downstream of aS playing a crucial role in its pathophysiology. This study describes the aS induced ubiquitination and degradation of the master stress-protective transcription factor, heat shock transcription factor 1 (HSF1), by Nedd4-1. In these studies, inhibiting Nedd4-1 with siRNA decreased the aS’s toxic effect in neuroblastoma. aS is not the only protein implicated in the pathophysiology of PD though. Shoshani et al. looks at RTP801, a stress regulated protein that when overexpress is sufficient to trigger cell death (Shoshani T. et al, 2002). RTP801 is upregulated in both sporadic and parkin mutant PD patients. This lab shows that Nedd4-1 expression reduces RTP801 mRNA and protein levels while Nedd4-1 inhibition causes toxicity mediated by RTP801 (Canal M. et al, 2016). Another couple studies describe a small-molecule NAB2 that reverses the pathology of PD and show Nedd4-1 mediating its mechanism of action (Chung CY. et al, 2013; Hatstat AK. et al, 2021).

II. Schizophrenia

Schizophrenia is a neurological disorder characterized by positive and negative cognitive and emotional symptoms. This disease has a strong familial component and is often studied using genetic tools. There are a few studies in the literature that suggest Nedd4-1’s involvement in the manifestation of this disease in the CNS. Warnica et al. was the first to implicate Nedd4-1 in their study assessing rare structural genetic changes (copy number

variants [CNVs]) in people with and without schizophrenia (Warnica W. et al, 2015). Gene-set enrichment analysis showed that predicted target genes of CNV overlapped miRNA included the NEDD4 gene along with other neuronal projection genes. Han et al. followed this study up by assessing the allele and genotype distributions of five loci of the NEDD4 gene in patients with and without schizophrenia using Taqman single-nucleotide polymorphism (SNP) genotyping technology (Han C. et al, 2019). They found that multiple of the NEDD4 loci were associated with the pathogenesis of schizophrenia and cognitive deficits of the disease. Another study used a similar technique to highlight an interaction between the NEDD4 gene and childhood trauma and its association with symptoms of Schizophrenia (Bi XJ. et al, 2021).

III. Cancer

Glioma is an aggressive and dangerous category of cancer that forms in the central nervous system. Nedd4-1 is implicated in the literature as a regulator in many types of cancer including Glioma. A majority of the work studying Nedd4-1's role in glioma describes Nedd4-1 ubiquitinating and degrading known tumor suppressor agents. Nedd4-1 is thought to regulate cellular transformation and tumor formation (PTEN, TUSC2) (Dai B. et al, 2010; Chuang HY. et al, 2021; Rimkus TK. et al, 2022) and cancer cell migration and invasion (CNrasGEF, LATS1) (Zhang H. et al, 2013; Ji J. et al, 2020) by degrading the tumor suppressors involved in those processes. Some of these studies go one step further and propose a regulatory mechanism for Nedd4-1. Dai et al. showed that overexpression the FoxM1B transcription factor up-regulated Nedd4-1 and subsequently down-regulated PTEN

levels in multiple cell lines (Dai B. et al, 2010). Ji et al. suggests that the transmembrane protein, prostate transmembrane protein, androgen induced 1 (PMEPA1) recruits Nedd4-1 to polyubiquitinate and target large tumor suppressor kinase 1 (LATS1) for degradation (Ji J. et al, 2020). What all these studies have in common is that Nedd4-1 expression shows a positive correlation for glioma tumor progression. Chuang et al. even uses Nedd4-1 activity inhibitor Indole-3-carbinol (I3C) to describe a crucial role for Nedd4-1 in Temozolomide (TMZ)-resistant glioblastoma (GBM) and that Nedd4-1 inhibition led to a re-sensitization of TMZ-resistant GBMs (Chuang HY. et al, 2021).

IV. Cerebral Ischemia

Cerebral ischemia is a byproduct of a restriction of blood flow or decrease oxygen levels in the brain. The mechanisms of this disorder remain largely unknown, but two studies in the literature suggest Nedd4-1's regulation of PTEN translocation as a likely mechanism. Studies using mouse models of cerebral ischemia were able to show that enhanced ubiquitination of PTEN was critical for nuclear translocation. Howitt et al. states that Nedd4 family–interacting protein 1 (Ndfip1) mediates Nedd4-1 ubiquitination of PTEN and that Ndfip1 overexpression drove PTEN nuclear import (Howitt J. et al, 2012). Dai et al. suggests another Nedd4-1 regulator to mediate PTEN translocation (Dai C. et al, 2021). This study shows that Nedd4-1 is required for α- and γ-adaptin binding protein (Aagab) mediated PTEN nuclear translocation, as is the mono ubiquitination of PTEN.

V. Neuropathic Pain

Neuropathic pain (NP) is characterized by spontaneous intermittent or ongoing pain that can be induced by nervous system damage caused by trauma or many different disorders. Botulinum toxin type A (BTX-A) was considered to be a new potential drug for neuropathic pain (NP) treatment and its mechanism is still not completely understood. One study shows that BTX-A reduces expression of pro-inflammatory factor SNAP23 by increasing Nedd4-1 expression (Wang X. et al, 2020). They demonstrate that Nedd4-1 binds to and ubiquitinates SNAP23 and may be regulated by TLR2/MyD88 pro-inflammatory factors.

F. Acknowledgements

Chapter 2, in whole, is currently being prepared for submission for publication of the material. Kevin White; Patrick Gentry N. The dissertation author was the primary investigator and author of this material.

G. References

Kumar, S., Tomooka, Y., & Noda, M. (1992). Identification of a set of genes with developmentally down-regulated expression in the mouse brain. Biochemical and biophysical research communications, 185(3), 1155–1161. https://doi.org/10.1016/0006-291x(92)91747-e.

Kumar, S., Harvey, K. F., Kinoshita, M., Copeland, N. G., Noda, M., & Jenkins, N. A. (1997). cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene. Genomics, 40(3), 435–443. https://doi.org/10.1006/geno.1996.4582

Anan, T., Nagata, Y., Koga, H., Honda, Y., Yabuki, N., Miyamoto, C., Kuwano, A., Matsuda, I., Endo, F., Saya, H., & Nakao, M. (1998). Human ubiquitin-protein ligase Nedd4: expression, subcellular localization and selective interaction with ubiquitin-conjugating enzymes. Genes to cells : devoted to molecular & cellular mechanisms, 3(11), 751–763. https://doi.org/10.1046/j.1365-2443.1998.00227.x

Plant, P. J., Yeger, H., Staub, O., Howard, P., & Rotin, D. (1997). The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca2+-dependent plasma membrane localization. The Journal of biological chemistry, 272(51), 32329–32336. https://doi.org/10.1074/jbc.272.51.32329

Plant, P. J., Lafont, F., Lecat, S., Verkade, P., Simons, K., & Rotin, D. (2000). Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb. The Journal of cell biology, 149(7), 1473–1484. https://doi.org/10.1083/jcb.149.7.1473

Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., & Rotin, D. (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. The EMBO journal, 15(10), 2371–2380.

Goulet, C. C., Volk, K. A., Adams, C. M., Prince, L. S., Stokes, J. B., & Snyder, P. M. (1998). Inhibition of the epithelial Na+ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. The Journal of biological chemistry, 273(45), 30012–30017. https://doi.org/10.1074/jbc.273.45.30012

Kanelis, V., Farrow, N. A., Kay, L. E., Rotin, D., & Forman-Kay, J. D. (1998). NMR studies of tandem WW domains of Nedd4 in complex with a PY motif-containing region of the epithelial sodium channel. Biochemistry and cell biology = Biochimie et biologie cellulaire, 76(2-3), 341–350. https://doi.org/10.1139/bcb-76-2-3-341

Abriel, H., Loffing, J., Rebhun, J. F., Pratt, J. H., Schild, L., Horisberger, J. D., Rotin, D., & Staub, O. (1999). Defective regulation of the epithelial Na+ channel by Nedd4 in

Liddle's syndrome. The Journal of clinical investigation, 103(5), 667–673. https://doi.org/10.1172/JCI5713

Farr, T. J., Coddington-Lawson, S. J., Snyder, P. M., & McDonald, F. J. (2000). Human Nedd4 interacts with the human epithelial Na+ channel: WW3 but not WW1 binds to Na+-channel subunits. The Biochemical journal, 345 Pt 3(Pt 3), 503–509.

Staub, O., Yeger, H., Plant, P. J., Kim, H., Ernst, S. A., & Rotin, D. (1997). Immunolocalization of the ubiquitin-protein ligase Nedd4 in tissues expressing the epithelial Na+ channel (ENaC). The American journal of physiology, 272(6 Pt 1), C1871–C1880. https://doi.org/10.1152/ajpcell.1997.272.6.C1871

Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., & Rotin, D. (1997). Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. The EMBO journal, 16(21), 6325–6336. https://doi.org/10.1093/emboj/16.21.6325

Harvey, K. F., Dinudom, A., Komwatana, P., Jolliffe, C. N., Day, M. L., Parasivam, G., Cook, D. I., & Kumar, S. (1999). All three WW domains of murine Nedd4 are involved in the regulation of epithelial sodium channels by intracellular Na+. The Journal of biological chemistry, 274(18), 12525–12530. https://doi.org/10.1074/jbc.274.18.12525

Jolliffe, C. N., Harvey, K. F., Haines, B. P., Parasivam, G., & Kumar, S. (2000). Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4. The Biochemical journal, 351 Pt 3(Pt 3), 557–565.

Dinudom, A., Harvey, K. F., Komwatana, P., Young, J. A., Kumar, S., & Cook, D. I. (1998). Nedd4 mediates control of an epithelial Na+ channel in salivary duct cells by cytosolic Na+. Proceedings of the National Academy of Sciences of the United States of America, 95(12), 7169–7173. https://doi.org/10.1073/pnas.95.12.7169

Abriel, H., Kamynina, E., Horisberger, J. D., & Staub, O. (2000). Regulation of the cardiac voltage-gated Na+ channel (H1) by the ubiquitin-protein ligase Nedd4. FEBS letters, 466(2-3), 377–380. https://doi.org/10.1016/s0014-5793(00)01098-x

Morrione, A., Plant, P., Valentinis, B., Staub, O., Kumar, S., Rotin, D., & Baserga, R. (1999). mGrb10 interacts with Nedd4. The Journal of biological chemistry, 274(34), 24094–24099. https://doi.org/10.1074/jbc.274.34.24094

Harvey, K. F., Harvey, N. L., Michael, J. M., Parasivam, G., Waterhouse, N., Alnemri, E. S., Watters, D., & Kumar, S. (1998). Caspase-mediated cleavage of the ubiquitin-protein ligase Nedd4 during apoptosis. The Journal of biological chemistry, 273(22), 13524–13530. https://doi.org/10.1074/jbc.273.22.13524.

Ikeda, M., Ikeda, A., Longan, L. C., & Longnecker, R. (2000). The Epstein-Barr virus latent membrane protein 2A PY motif recruits WW domain-containing ubiquitin-protein ligases. Virology, 268(1), 178–191. https://doi.org/10.1006/viro.1999.0166

Winberg, G., Matskova, L., Chen, F., Plant, P., Rotin, D., Gish, G., Ingham, R., Ernberg, I., & Pawson, T. (2000). Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. Molecular and cellular biology, 20(22), 8526–8535. https://doi.org/10.1128/MCB.20.22.8526-8535.2000

Strack, B., Calistri, A., Accola, M. A., Palu, G., & Gottlinger, H. G. (2000). A role for ubiquitin ligase recruitment in retrovirus release. Proceedings of the National Academy of Sciences of the United States of America, 97(24), 13063–13068. https://doi.org/10.1073/pnas.97.24.13063

Harty, R. N., Brown, M. E., Wang, G., Huibregtse, J., & Hayes, F. P. (2000). A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. Proceedings of the National Academy of Sciences of the United States of America, 97(25), 13871–13876. https://doi.org/10.1073/pnas.250277297

Kamynina, E., Debonneville, C., Bens, M., Vandewalle, A., & Staub, O. (2001). A novel mouse Nedd4 protein suppresses the activity of the epithelial Na+ channel. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 15(1), 204–214. https://doi.org/10.1096/fj.00-0191com

Fotia AB, Ekberg J, Adams DJ, Cook DI, Poronnik P, Kumar S. Regulation of neuronal voltage-gated sodium channels by the ubiquitin-protein ligases Nedd4 and Nedd4-2. J Biol Chem. 2004;279(28):28930-28935. doi:10.1074/jbc.M402820200

Scheffner M, Kumar S. Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. Biochim. Biophys. Acta. 2014;1843:61–74. doi: 10.1016/j.bbamcr.2013.03.024.

Huang X, Chen J, Cao W, Yang L, Chen Q, He J, Yi Q, Huang H, Zhang E, Cai Z. The many substrates and functions of NEDD4-1. Cell Death Dis. 2019;10(12):904. Published 2019 Dec 2. doi:10.1038/s41419-019-2142-8

Dunn R, Klos DA, Adler AS, Hicke L. The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. J Cell Biol. 2004;165(1):135-144. doi:10.1083/jcb.200309026

Boase NA, Kumar S. NEDD4: The founding member of a family of ubiquitin-protein ligases. Gene. 2015;557(2):113-122. doi:10.1016/j.gene.2014.12.020

Sudol M, Chen HI, Bougeret C, Einbond A, Bork P. Characterization of a novel protein-binding module--the WW domain. FEBS Lett. 1995;369(1):67-71. doi:10.1016/0014-5793(95)00550-s

Dodson EJ, Fishbain-Yoskovitz V, Rotem-Bamberger S, Schueler-Furman O. Versatile communication strategies among tandem WW domain repeats. Exp Biol Med (Maywood). 2015;240(3):351-360. doi:10.1177/1535370214566558

Rotin D, Kumar S. Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol. 2009;10(6):398-409. doi:10.1038/nrm2690

Boase NA, Kumar S. NEDD4: The founding member of a family of ubiquitin-protein ligases. Gene. 2015;557(2):113-122. doi:10.1016/j.gene.2014.12.020

Chen C, Matesic LE. The Nedd4-like family of E3 ubiquitin ligases and cancer. Cancer Metastasis Rev. 2007;26(3-4):587-604. doi:10.1007/s10555-007-9091-x

Zhou W, Xu J, Zhao Y, Sun Y. SAG/RBX2 is a novel substrate of NEDD4-1 E3 ubiquitin ligase and mediates NEDD4-1 induced chemosensitization. Oncotarget. 2014;5(16):6746-6755. doi:10.18632/oncotarget.2246

Liu Y, Oppenheim RW, Sugiura Y, Lin W. Abnormal development of the neuromuscular junction in Nedd4-deficient mice. Dev Biol. 2009;330(1):153-166. doi:10.1016/j.ydbio.2009.03.023

Camera D, Boase NA, Kumar S, Pow DV, Poronnik P. Subtle gait abnormalities in Nedd4 heterozygous mice. Behav Brain Res. 2014;260:15-24. doi:10.1016/j.bbr.2013.11.024

Camera D, Coleman HA, Parkington HC, Jenkins TA, Pow DV, Boase N, Kumar S, Poronnik P. Learning, memory and long-term potentiation are altered in Nedd4 heterozygous mice. Behav Brain Res. 2016;303:176-181. doi:10.1016/j.bbr.2016.01.054

Kawabe H, Neeb A, Dimova K, Young SM Jr, Takeda M, Katsurabayashi S, Mitkovski M, Malakhova OA, Zhang DE, Umikawa M, Kariya K, Goebbels S, Nave KA, Rosenmund C, Jahn O, Rhee J, Brose N. Regulation of Rap2A by the ubiquitin ligase Nedd4-1 controls neurite development. Neuron. 2010;65(3):358-372. doi:10.1016/j.neuron.2010.01.007

Fu Z, Lee SH, Simonetta A, Hansen J, Sheng M, Pak DT. Differential roles of Rap1 and Rap2 small GTPases in neurite retraction and synapse elimination in hippocampal spiny neurons. J Neurochem. 2007;100(1):118-131. doi:10.1111/j.1471-4159.2006.04195.x

Taira K, Umikawa M, Takei K, Myagmar BE, Shinzato M, Machida N, Uezato H, Nonaka S, Kariya K. The Traf2- and Nck-interacting kinase as a putative effector of Rap2 to regulate actin cytoskeleton. J Biol Chem. 2004;279(47):49488-49496. doi:10.1074/jbc.M406370200

Kawabe H, Neeb A, Dimova K, Young SM Jr, Takeda M, Katsurabayashi S, Mitkovski M, Malakhova OA, Zhang DE, Umikawa M, Kariya K, Goebbels S, Nave KA, Rosenmund C, Jahn O, Rhee J, Brose N. Regulation of Rap2A by the ubiquitin ligase Nedd4-1 controls neurite development. Neuron. 2010;65(3):358-372. doi:10.1016/j.neuron.2010.01.007

Christie KJ, Martinez JA, Zochodne DW. Disruption of E3 ligase NEDD4 in peripheral neurons interrupts axon outgrowth: Linkage to PTEN. Mol Cell Neurosci. 2012;50(2):179-192. doi:10.1016/j.mcn.2012.04.006

Hsia HE, Kumar R, Luca R, Takeda M, Courchet J, Nakashima J, Wu S, Goebbels S, An W, Eickholt BJ, Polleux F, Rotin D, Wu H, Rossner MJ, Bagni C, Rhee JS, Brose N, Kawabe H. Ubiquitin E3 ligase Nedd4-1 acts as a downstream target of PI3K/PTEN-mTORC1 signaling to promote neurite growth. Proc Natl Acad Sci U S A. 2014;111(36):13205-13210. doi:10.1073/pnas.1400737111

Sakakibara M, Uenoyama Y, Minabe S, Watanabe Y, Deura C, Nakamura S, Suzuki G, Maeda K, Tsukamura H. Microarray analysis of perinatal-estrogen-induced changes in gene expression related to brain sexual differentiation in mice. PLoS One. 2013;8(11):e79437. Published 2013 Nov 4. doi:10.1371/journal.pone.0079437

Shepherd JD, Huganir RL. The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol. 2007;23:613-643. doi:10.1146/annurev.cellbio.23.090506.123516

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Lin A, Hou Q, Jarzylo L, Amato S, Gilbert J, Shang F, Man HY. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. J Neurochem. 2011;119(1):27-39. doi:10.1111/j.1471-4159.2011.07221.x

Hou Q, Gilbert J, Man HY. Homeostatic regulation of AMPA receptor trafficking and degradation by light-controlled single-synaptic activation. Neuron. 2011;72(5):806-818. doi:10.1016/j.neuron.2011.10.011

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Wei J, Xiong Z, Lee JB, Cheng J, Duffney LJ, Matas E, Yan Z. Histone Modification of Nedd4 Ubiquitin Ligase Controls the Loss of AMPA Receptors and Cognitive Impairment Induced by Repeated Stress. J Neurosci. 2016;36(7):2119-2130. doi:10.1523/JNEUROSCI.3056-15.2016

Gautam V, Trinidad JC, Rimerman RA, Costa BM, Burlingame AL, Monaghan DT. Nedd4 is a specific E3 ubiquitin ligase for the NMDA receptor subunit GluN2D. Neuropharmacology. 2013;74:96-107. doi:10.1016/j.neuropharm.2013.04.035

Lee S, Park S, Lee H, Han S, Song JM, Han D, Suh YH. Nedd4 E3 ligase and beta-arrestins regulate ubiquitination, trafficking, and stability of the mGlu7 receptor. Elife. 2019;8:e44502. Published 2019 Aug 2. doi:10.7554/eLife.44502

Persaud A, Alberts P, Hayes M, Guettler S, Clarke I, Sicheri F, Dirks P, Ciruna B, Rotin D. Nedd4-1 binds and ubiquitylates activated FGFR1 to control its endocytosis and function. EMBO J. 2011;30(16):3259-3273. Published 2011 Jul 15. doi:10.1038/emboj.2011.234

Persaud A, Alberts P, Mari S, Tong J, Murchie R, Maspero E, Safi F, Moran MF, Polo S, Rotin D. Tyrosine phosphorylation of NEDD4 activates its ubiquitin ligase activity. Sci Signal. 2014;7(346):ra95. Published 2014 Oct 7. doi:10.1126/scisignal.2005290

Giaume C, Theis M. Pharmacological and genetic approaches to study connexin-mediated channels in glial cells of the central nervous system. Brain Res Rev. 2010;63(1-2):160-176. doi:10.1016/j.brainresrev.2009.11.005

Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N. Astroglial networks: a step further in neuroglial and gliovascular interactions. Nat Rev Neurosci. 2010;11(2):87-99. doi:10.1038/nrn2757

Liao CK, Jeng CJ, Wang HS, Wang SH, Wu JC. Lipopolysaccharide induces degradation of connexin43 in rat astrocytes via the ubiquitin-proteasome proteolytic pathway. PLoS One. 2013;8(11):e79350. Published 2013 Nov 13. doi:10.1371/journal.pone.0079350

Uchiyama M, Nakao A, Kurita Y, Fukushi I, Takeda K, Numata T, Tran HN, Sawamura S, Ebert M, Kurokawa T, Sakaguchi R, Stokes AJ, Takahashi N, Okada Y, Mori Y. O2-Dependent Protein Internalization Underlies Astrocytic Sensing of Acute Hypoxia by Restricting Multimodal TRPA1 Channel Responses. Curr Biol. 2020;30(17):3378-3396.e7. doi:10.1016/j.cub.2020.06.047

Duncan CE, Chetcuti AF, Schofield PR. Coregulation of genes in the mouse brain following treatment with clozapine, haloperidol, or olanzapine implicates altered potassium channel subunit expression in the mechanism of antipsychotic drug action. Psychiatr Genet. 2008;18(5):226-239. doi:10.1097/YPG.0b013e3283053019

Stockton SD Jr, Gomes I, Liu T, Moraje C, Hipólito L, Jones MR, Ma'ayan A, Morón JA, Li H, Devi LA. Morphine Regulated Synaptic Networks Revealed by Integrated Proteomics and Network Analysis. Mol Cell Proteomics. 2015;14(10):2564-2576. doi:10.1074/mcp.M115.047977

Dugger BN, Dickson DW. Pathology of Neurodegenerative Diseases. Cold Spring Harb Perspect Biol. 2017;9(7):a028035. Published 2017 Jul 5. doi:10.1101/cshperspect.a028035

Kwak YD, Wang B, Li JJ, Wang R, Deng Q, Diao S, Chen Y, Xu R, Masliah E, Xu H, Sung JJ, Liao FF. Upregulation of the E3 ligase NEDD4-1 by oxidative stress degrades IGF-1 receptor protein in neurodegeneration. J Neurosci. 2012;32(32):10971-10981. doi:10.1523/JNEUROSCI.1836-12.2012

Oboudiyat C, Glazer H, Seifan A, Greer C, Isaacson RS. Alzheimer's disease. Semin Neurol. 2013;33(4):313-329. doi:10.1055/s-0033-1359319

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Akkaya BG, Zolnerciks JK, Ritchie TK, Bauer B, Hartz AM, Sullivan JA, Linton KJ. The multidrug resistance pump ABCB1 is a substrate for the ubiquitin ligase NEDD4-1. Mol Membr Biol. 2015;32(2):39-45. doi:10.3109/09687688.2015.1023378

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Akkaya BG, Zolnerciks JK, Ritchie TK, Bauer B, Hartz AM, Sullivan JA, Linton KJ. The multidrug resistance pump ABCB1 is a substrate for the ubiquitin ligase NEDD4-1. Mol Membr Biol. 2015;32(2):39-45. doi:10.3109/09687688.2015.1023378

Chai AB, Callaghan R, Gelissen IC. The Ubiquitin E3 Ligase Nedd4 Regulates the Expression and Amyloid-β Peptide Export Activity of P-Glycoprotein. Int J Mol Sci. 2022;23(3):1019. Published 2022 Jan 18. doi:10.3390/ijms23031019

Akkaya BG, Zolnerciks JK, Ritchie TK, Bauer B, Hartz AM, Sullivan JA, Linton KJ. The multidrug resistance pump ABCB1 is a substrate for the ubiquitin ligase NEDD4-1. Mol Membr Biol. 2015;32(2):39-45. doi:10.3109/09687688.2015.1023378

Correani V, Martire S, Mignogna G, Caruso LB, Tempera I, Giorgi A, Grieco M, Mosca L, Schininà ME, Maras B, d'Erme M. Poly(ADP-ribosylated) proteins in β-amyloid peptide-stimulated microglial cells. Biochem Pharmacol. 2019;167:50-57. doi:10.1016/j.bcp.2018.10.026

Dorsey ER, Bloem BR. The Parkinson Pandemic-A Call to Action. JAMA Neurol. 2018;75(1):9-10. doi:10.1001/jamaneurol.2017.3299

Jankovic J, Tan EK. Parkinson's disease: etiopathogenesis and treatment. J Neurol Neurosurg Psychiatry. 2020;91(8):795-808. doi:10.1136/jnnp-2019-322338

Goedert M, Spillantini MG, Del Tredici K, Braak H. 100 years of Lewy pathology. Nat Rev Neurol. 2013;9(1):13-24. doi:10.1038/nrneurol.2012.242

Tofaris GK, Kim HT, Hourez R, Jung JW, Kim KP, Goldberg AL. Ubiquitin ligase Nedd4 promotes alpha-synuclein degradation by the endosomal-lysosomal pathway. Proc Natl Acad Sci U S A. 2011;108(41):17004-17009. doi:10.1073/pnas.1109356108 https://doi.org/10.1073/pnas.1109356108

Sugeno N, Hasegawa T, Tanaka N, Fukuda M, Wakabayashi K, Oshima R, Konno M, Miura E, Kikuchi A, Baba T, Anan T, Nakao M, Geisler S, Aoki M, Takeda A. Lys-63-linked ubiquitination by E3 ubiquitin ligase Nedd4-1 facilitates endosomal sequestration of internalized α-synuclein. J Biol Chem. 2014;289(26):18137-18151. doi:10.1074/jbc.M113.529461

Mund T, Masuda-Suzukake M, Goedert M, Pelham HR. Ubiquitination of alpha-synuclein filaments by Nedd4 ligases [published correction appears in PLoS One. 2018 Aug 7;13(8):e0202158]. PLoS One. 2018;13(7):e0200763. Published 2018 Jul 18. doi:10.1371/journal.pone.0200763

Won SY, Park JJ, You ST, Hyeun JA, Kim HK, Jin BK, McLean C, Shin EY, Kim EG. p21-activated kinase 4 controls the aggregation of α-synuclein by reducing the monomeric and aggregated forms of α-synuclein: involvement of the E3 ubiquitin ligase NEDD4-1. Cell Death Dis. 2022;13(6):575. Published 2022 Jun 30. doi:10.1038/s41419-022-05030-1

Tofaris GK, Kim HT, Hourez R, Jung JW, Kim KP, Goldberg AL. Ubiquitin ligase Nedd4 promotes alpha-synuclein degradation by the endosomal-lysosomal pathway. Proc Natl Acad Sci U S A. 2011;108(41):17004-17009. doi:10.1073/pnas.1109356108 https://doi.org/10.1073/pnas.1109356108 https://doi.org/10.1073/pnas.1109356108

Davies SE, Hallett PJ, Moens T, Smith G, Mangano E, Kim HT, Goldberg AL, Liu JL, Isacson O, Tofaris GK. Enhanced ubiquitin-dependent degradation by Nedd4 protects against α-synuclein accumulation and toxicity in animal models of Parkinson's disease. Neurobiol Dis. 2014;64(100):79-87. doi:10.1016/j.nbd.2013.12.011

Sugeno N, Hasegawa T, Tanaka N, Fukuda M, Wakabayashi K, Oshima R, Konno M, Miura E, Kikuchi A, Baba T, Anan T, Nakao M, Geisler S, Aoki M, Takeda A. Lys-63-linked ubiquitination by E3 ubiquitin ligase Nedd4-1 facilitates endosomal sequestration of internalized α-synuclein. J Biol Chem. 2014;289(26):18137-18151. doi:10.1074/jbc.M113.529461

Mund T, Masuda-Suzukake M, Goedert M, Pelham HR. Ubiquitination of alpha-synuclein filaments by Nedd4 ligases [published correction appears in PLoS One. 2018 Aug 7;13(8):e0202158]. PLoS One. 2018;13(7):e0200763. Published 2018 Jul 18. doi:10.1371/journal.pone.0200763

Vogiatzis S, Celestino M, Trevisan M, Magro G, Del Vecchio C, Erdengiz D, Palù G, Parolin C, Maguire-Zeiss K, Calistri A. Lentiviral Vectors Expressing Chimeric NEDD4 Ubiquitin Ligases: An Innovative Approach for Interfering with Alpha-Synuclein Accumulation. Cells. 2021;10(11):3256. Published 2021 Nov 21. doi:10.3390/cells10113256

Kim E, Wang B, Sastry N, Masliah E, Nelson PT, Cai H, Liao FF. NEDD4-mediated HSF1 degradation underlies α-synucleinopathy. Hum Mol Genet. 2016;25(2):211-222. doi:10.1093/hmg/ddv445

Shoshani T, Faerman A, Mett I, Zelin E, Tenne T, Gorodin S, Moshel Y, Elbaz S, Budanov A, Chajut A, Kalinski H, Kamer I, Rozen A, Mor O, Keshet E, Leshkowitz D, Einat P, Skaliter R, Feinstein E. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Mol Cell Biol. 2002;22(7):2283-2293. doi:10.1128/MCB.22.7.2283-2293.2002

Canal M, Martín-Flores N, Pérez-Sisqués L, Romaní-Aumedes J, Altas B, Man HY, Kawabe H, Alberch J, Malagelada C. Loss of NEDD4 contributes to RTP801 elevation and neuron toxicity: implications for Parkinson's disease. Oncotarget. 2016;7(37):58813-58831. doi:10.18632/oncotarget.11020

Chung CY, Khurana V, Auluck PK, Tardiff DF, Mazzulli JR, Soldner F, Baru V, Lou Y, Freyzon Y, Cho S, Mungenast AE, Muffat J, Mitalipova M, Pluth MD, Jui NT, Schüle B, Lippard SJ, Tsai LH, Krainc D, Buchwald SL, Jaenisch R, Lindquist S. Identification and rescue of α-synuclein toxicity in Parkinson patient-derived neurons. Science. 2013;342(6161):983-987. doi:10.1126/science.1245296

Hatstat AK, Ahrendt HD, Foster MW, Mayne L, Moseley MA, Englander SW, McCafferty DG. Characterization of Small-Molecule-Induced Changes in Parkinson's-Related Trafficking via the Nedd4 Ubiquitin Signaling Cascade. Cell Chem Biol. 2021;28(1):14-25.e9. doi:10.1016/j.chembiol.2020.10.008

Warnica W, Merico D, Costain G, Alfred SE, Wei J, Marshall CR, Scherer SW, Bassett AS. Copy number variable microRNAs in schizophrenia and their neurodevelopmental gene targets. Biol Psychiatry. 2015;77(2):158-166. doi:10.1016/j.biopsych.2014.05.011

Han C, Cui K, Bi X, Wang L, Sun M, Yang L, Liu L. Association between polymorphism of the NEDD4 gene and cognitive dysfunction of schizophrenia patients in Chinese Han population. BMC Psychiatry. 2019;19(1):405. Published 2019 Dec 18. doi:10.1186/s12888-019-2386-y

Bi XJ, Hu L, Qiao DD, Han C, Sun MM, Cui KY, Wang LN, Yang LM, Liu LF, Chen ZY. Evidence for an Interaction Between NEDD4 and Childhood Trauma on Clinical Characters of Schizophrenia With Family History of Psychosis. Front Psychiatry. 2021;12:608231. Published 2021 Apr 8. doi:10.3389/fpsyt.2021.608231

Dai B, Pieper RO, Li D, Wei P, Liu M, Woo SY, Aldape KD, Sawaya R, Xie K, Huang S. FoxM1B regulates NEDD4-1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. Cancer Res. 2010;70(7):2951-2961. doi:10.1158/0008-5472.CAN-09-3909

Chuang HY, Hsu LY, Pan CM, Pikatan NW, Yadav VK, Fong IH, Chen CH, Yeh CT, Chiu SC. The E3 Ubiquitin Ligase NEDD4-1 Mediates Temozolomide-Resistant Glioblastoma through PTEN Attenuation and Redox Imbalance in Nrf2-HO-1 Axis. Int J Mol Sci. 2021;22(19):10247. Published 2021 Sep 23. doi:10.3390/ijms221910247

Rimkus TK, Arrigo AB, Zhu D, Carpenter RL, Sirkisoon S, Doheny D, Regua AT, Wong GL, Manore S, Wagner C, Lin HK, Jin G, Ruiz J, Chan M, Debinski W, Lo HW. NEDD4 degrades TUSC2 to promote glioblastoma progression. Cancer Lett. 2022;531:124-135. doi:10.1016/j.canlet.2022.01.029

Zhang H, Nie W, Zhang X, Zhang G, Li Z, Wu H, Shi Q, Chen Y, Ding Z, Zhou X, Yu R. NEDD4-1 regulates migration and invasion of glioma cells through CNrasGEF ubiquitination in vitro. PLoS One. 2013;8(12):e82789. Published 2013 Dec 10. doi:10.1371/journal.pone.0082789

Ji J, Ding K, Luo T, Xu R, Zhang X, Huang B, Chen A, Zhang D, Miletic H, Bjerkvig R, Thorsen F, Wang J, Li X. PMEPA1 isoform a drives progression of glioblastoma by promoting protein degradation of the Hippo pathway kinase LATS1. Oncogene. 2020;39(5):1125-1139. doi:10.1038/s41388-019-1050-9

Dai B, Pieper RO, Li D, Wei P, Liu M, Woo SY, Aldape KD, Sawaya R, Xie K, Huang S. FoxM1B regulates NEDD4-1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. Cancer Res. 2010;70(7):2951-2961. doi:10.1158/0008-5472.CAN-09-3909

Ji J, Ding K, Luo T, Xu R, Zhang X, Huang B, Chen A, Zhang D, Miletic H, Bjerkvig R, Thorsen F, Wang J, Li X. PMEPA1 isoform a drives progression of glioblastoma by

promoting protein degradation of the Hippo pathway kinase LATS1. Oncogene. 2020;39(5):1125-1139. doi:10.1038/s41388-019-1050-9

Chuang HY, Hsu LY, Pan CM, Pikatan NW, Yadav VK, Fong IH, Chen CH, Yeh CT, Chiu SC. The E3 Ubiquitin Ligase NEDD4-1 Mediates Temozolomide-Resistant Glioblastoma through PTEN Attenuation and Redox Imbalance in Nrf2-HO-1 Axis. Int J Mol Sci. 2021;22(19):10247. Published 2021 Sep 23. doi:10.3390/ijms221910247

Howitt J, Lackovic J, Low LH, Naguib A, Macintyre A, Goh CP, Callaway JK, Hammond V, Thomas T, Dixon M, Putz U, Silke J, Bartlett P, Yang B, Kumar S, Trotman LC, Tan SS. Ndfip1 regulates nuclear Pten import in vivo to promote neuronal survival following cerebral ischemia. J Cell Biol. 2012;196(1):29-36. doi:10.1083/jcb.201105009

Dai C, Wu B, Chen Y, Li X, Bai Y, Du Y, Pang Y, Wang YT, Dong Z. Aagab acts as a novel regulator of NEDD4-1-mediated Pten nuclear translocation to promote neurological recovery following hypoxic-ischemic brain damage. Cell Death Differ. 2021;28(8):2367-2384. doi:10.1038/s41418-021-00757-4

Wang X, Tian S, Wang H, Liu P, Zheng H, Wu L, Liu Q, Wu W. Botulinum toxin type A alleviates neuropathic pain and suppresses inflammatory cytokines release from microglia by targeting TLR2/MyD88 and SNAP23. Cell Biosci. 2020;10(1):141. Published 2020 Dec 9. doi:10.1186/s13578-020-00501-4

Chapter III

Glutamatergic signaling drives differentiated Nedd4-1 trafficking dynamics

A. Abstract

Post-mitotic neurons have unique demands when it comes to cellular function due to their long processes and a need for dynamic response to external stimuli. Therefore, it is important for neurons to be able to finely regulate the subcellular localization of their proteins. One such protein whose subcellular distribution may be rate-limiting in its function is the ubiquitin E3 ligase Nedd4-1. Nedd4-1 plays a critical role in protein homeostasis, among other things, and has been shown to be important for normal neuronal function and may contribute to the pathophysiology of many neurological and neurodegenerative disorders. Yet, much is not understood about the dynamic regulation of the subcellular localization of Nedd4-1. We utilize a fluorescently tagged Nedd4-1 construct to build a live time-lapse imaging platform to study the rules regulating trafficking of Nedd4-1. We successfully validate the functionality of our GFP-Nedd4-1 viral-expression construct for capture of dynamic live imaging of activity dependent translocation of Nedd4-1. Furthermore, we show that independently of each other AMPAR and NMDAR stimulation drive Nedd4-1 drive varied trafficking dynamics of the Nedd4-1. We see differences in cell latency to redistribution, average puncta velocity, puncta size, and direction of movement. Our results indicate that we can confidently study the dynamic subcellular localization of an E3 ligase in live neurons and that Nedd4-1 trafficking is regulated at a fine level.

B. Introduction

Eukaryotic cells host upwards of 10,000 different proteins present in varying abundances. Each of these proteins perform very specialized roles with the cell. For this

reason, cells tend to be very compartmentalized to allow for a variety of tasks necessary to maintain homeostasis. Because protein function is incredibly influenced by subcellular localization (such as chemical environment and potential interactors), it is important to finely regulate protein localization. This is especially true for neuronal cell types that can have incredibly long processes and need to dynamically respond to changes in environmental stimuli. This necessitates a complex cytoskeletal machinery that allows for long range and dynamic trafficking of proteins. Many different biological processes require active translocation of proteins, and the dysregulation of this trafficking is thought to underlie several neurological disorders and cell health issues. Understanding the dynamic trafficking of proteins within the neuron is essential to understanding neuronal function in the central nervous system.

One of these closely regulated proteins is Nedd4-1. Nedd4-1 operates within the ubiquitin-proteasome system (UPS) and has been shown to play a significant role in neuronal health. The UPS is built of three parts – the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ligating enzyme (ubiquitin ligase) – that function to transport ubiquitin molecules and attach them to lysine residues on their substrates. Monoubiquitination (1 ubiquitin modification) and polyubiquitination (2 or more chains of ubiquitin modifications) are used to regulate modified substrates to degradation in the proteasome/lysosome or to non-degradative pathways. Through ubiquitin modification, Nedd4-1 plays a role in motor development, axon branching/neurite growth, receptor regulation, gap junction formation, and O_2 homeostasis. Part of Nedd4-1's ability to engage in a diversity of biological pathways within the neuron arises from its finely regulated subcellular localization. The more we can

uncover about the regulation of this protein's localization, the more we will understand about Nedd4-1's function in neuronal cell health.

A lot can be understood about the regulation of Nedd4-1's subcellular localization through the structure of ligase. Nedd4-1 consists of three separate functional domains: (C2) calcium binding domain, various (WW) tryptophan substrate binding domains, and (HECT) catalytic domain. The HECT domain is Nedd4-1's functional domain that receives ubiquitin molecules and catalyzes the bond between ubiquitin and its lysine binding site. This domain likely has very little to do with Nedd4-1 translocation. The WW domains, on the other hand, are the substrate binding domains. These domains are known to recognize proline rich PPxY regions on target substrates to bind to and facilitate ubiquitination – although many Nedd4-1 substrates have been discovered that do not contain these proline rich regions. The WW domain likely plays a larger role in Nedd4-1 translocation if not for the simple fact that they must be spatially near their target substrates to successfully bind to them. By far the most important functional domain to the trafficking of Nedd4-1 is its C2 domain. Nedd4-1's C2 domain functions similarly to that of PKCα's and binds to Ca^{2+} and Phospholipid membranes. The C2 domains affinity for phospholipid membranes allows the ligase to traffic to the cell membrane or associate with membrane bound organelles in the cytoplasm. Its affinity to Ca^{2+} also allows the ligase to be sensitive to Ca^{2+} changes in its environment. Together these allow for a dynamic trafficking of the Nedd4-1 protein.

In addition to dynamic translocation in the cell, Nedd4-1 is one of the few E3 ligases shown to traffic in an activity dependent manner. Our lab was the first but not the only to

show that glutamatergic stimulation caused Nedd4-1 to go from a diffuse distribution to cluster together into puncta that were then recruited to the synapse (Scudder SL. et al, 2014; Lin A. et al, 2011). Previous research had already described Nedd4-1 as a crucial component of AMPA-dependent ubiquitination of AMPA receptors (AMPARs) (Schwarz LA. et al, 2010). The activity-dependent redistribution of the ligase supported these claims. This phenotype was not only described in healthy neurons but also as a possible mechanism of Aβ induced downregulation of functional AMPARs. Aβ treated cells showed similar redistribution of Nedd4-1 and knockdown of the ligase rescued synaptic function dysregulated by Aβ treatment (Rodrigues EM. et al, 2016). Together these findings provide evidence of the importance of regulating the subcellular localization of Nedd4-1. In this chapter, we explore the rules regulating Nedd4-1 trafficking dynamics to better understand its function and dysfunction in neurons.

C. Materials and Methods

Antibodies and reagents. Antibodies were as follows: pAb GFP (Invitrogen), mAb HA (BioLegend), pAb MAP2 (Abcam), mAb Nedd4-1 (Biosciences), mAb Tubulin (Sigma-Aldrich), mAb PSD-95 (EMD Millipore). Reagents were as follows: Glutamate, Glycine, AMPA, NMDA, CNQX, DL-2-amino-5-phosphonopentanoic acid (APV).

DNAconstructs. The attenuated sindbis GFP-tagged Nedd4-1 construct was created by cloning the GFP upstream of mouse Nedd4-1(with a small truncated N-terminus; delta110) sequence in pcDNA3.1 resuling in pCDNA3.1-GFP-Nedd4-1 construct. GFP-Nedd4-1 was then cloned into attenuated pSinRep5. Production of recombinant Sindbis virus was

performed as described previously (Djakovic SN. et al., 2009). All DNA and viral constructs were verified by sequencing.

Neuron cultures and infections. Rat dissociated hippocampal or cortical neurons from postnatal day 1 pups of either sex were plated at a density of 45,000 cells/cm2 onto poly-d-lysine-coated coverslips, poly-d-lysine-coated 6-well plastic dishes at ~500,000 cells per well (cortical cultures), or poly-d-lysine-coated 10cm plastic dishes at ~3,000,000 cells dish (cortical cultures). These cultures were maintained in B27 supplemented neurobasal medium (Invitrogen) until ≥18 d in vitro (DIV), as described previously (Scudder SL. et al. 2014, Djakovic SN. et al., 2009; Schwarz LA. et al., 2010; Djakovic SN. et al., 2012). Hippocampal or cortical cultures were infected with attenuated Sindbis virion at DIV 18–21 and expression was limited to 16–20 h to ensure cell health as in previous studies (Djakovic SN. et al., 2012; Scudder SL. et al., 2014).

Immunostaining. After infections and drug treatments, neurons were washed with cold PBS-MC and fixed with a solution containing 4% paraformaldehyde and 4% sucrose for 10 min. Cells were then permeabilized with 0.2% Triton X-100 and 2% BSA in PBS-MC for 20 min, followed by a 1 h block in 5% BSA in PBS-MC. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 12-20 h at 4°C or 1 h at room temperature respectively. Coverslips were mounted onto glass slides for confocal imaging.

Confocal Imaging (static and live imaging). All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nipkon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 μm/pixel at 1×),

Plan Apochromat 63×/1.4 numerical aperture objective, and an argon/krypton 100 mW air-cooled laser for 488/568/647 nm excitations. Maximum projected confocal Z-stacks were analyzed with ImageJ. For live imaging, cultures were placed in a humidified chamber maintained at 37°C. For live imaging, cells were transferred to either HBS or Hibernate-E low-fluorescence medium (BrainBits) at 37°C. Single z-planes were captured using a 40× or 63× objective with consistent imaging parameters (200ms exposure with images taken every 15sec for a duration of 10min).

Image Analysis. For redistribution experiments (static and live), a macro was used to straighten dendrites, crop a 50 μm segment 25 μm from the cell body, and randomize the file names. A custom macro was used to assist in the blinded manual counting of puncta. Quantification of percent of redistributed cells was done by visual analysis of whole cells for a consistent threshold of redistribution. For live imaging experiments open-source software, Kymographclear and Kymograph direct (Mangeol P. et al, 2016), were used to generate kymographs of selected dendrites, identify particles along the dendrite, and quantify the mobility of those particles (velocity over time, direction of particle movement). Particle size was calculated using ImageJ. In each dendritic segment, a minimum threshold value was determined by measuring the dimmest viable puncta in that segment and used to threshold that image. That image was then analyzed using the ImageJ macro Analyze Particles to determine the size of the particles. Statistical significance was determined through unpaired t tests or ANOVA with specified post hoc multiple-comparisons test using Prism software (GraphPad) or using a two-population proportion test for percent of redistributed cells data.

Western Blots. 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 incubating for 20 min at 4°C. Protein concentration was determined by BCA protein assay (Pierce) and equal protein amounts were loaded. Samples were boiled with sample buffer, resolved on 8% SDS page, and probed with primary antibodies. Blots were digitized and band intensities quantitated using ImageJ. For quantification target protein levels after treatment of neuronal cultures, band intensities in each condition were normalized to tubulin band mean intensity from the same sample.

Co-Immunoprecipitations. Cultured rat cortical neurons were lysed in buffer (50 mm Tric-HCl, 150 mm NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors. Homogenates were cleared by centrifugation at 14,000 rpm at 4°C. For immunoprecipitations (IPs), cleared lysates were incubated with primary antibodies at 4°C overnight, after which protein A or protein G agarose beads were added for an additional 1 h (Pierce). Immunoprecipitates were then washed, boiled in sample buffer, resolved on SDS PAGE, and probed in Western blot analysis.

D. Results

Both AMPA and NMDA receptor activation independently drive the redistribution of Nedd4-1.

It has been shown previously that the Nedd4-1 can be regulated in an activity dependent manner to ubiquitinate the GluA1 subunit of AMPA receptors (Scudder SL. et al,

2014). Previous experiments have described the activity dependent regulation of Nedd4-1 to be NMDA receptor independent. This was a conundrum in the field since it is known that NMDARs play a significant role in the trafficking of AMPARs to and from the synapse (Shepherd and Huganir, 2007). We decided to better understand the activity-dependent trafficking dynamics of Nedd4-1 in neurons using GFP-tagged Nedd4-1 and live time-lapse imaging. To visualize the cellular distribution of Nedd4-1 under various conditions, we first used immunocytochemistry in dissociated hippocampal neurons expressing GFP-tagged Nedd4-1 by attenuated Sindbis (limiting expression to 16-20 h) (**Fig. 1** A-C). Previous experiments have used an HA-tagged Nedd4-1 to observe its redistribution (Scudder et al., 2014). Although, GFP is larger than the HA-tag, expression of both tagged version of Nedd4-1 were similar and we found that the GFP-Nedd4-1 rapidly redistributed from diffuse a punctate distribution in response to glutamate similar to HA-Nedd4-1 redistribution in response to AMPA (**Fig 2** A, B). Furthermore, we observed a drastic change in the distribution of GFP-Nedd4-1 in AMPA treated cells (10 μM for 10 min) when compared to untreated cells as previously described ($p<0.001$, n=150; **Fig. 3** A, B). The number of redistributed cells is quantified using confocal imaging (See Materials and Methods). Additionally, we find that cells treated with NMDA on a concentration gradient (0.5 μM – 50 μM) (n=100) shows increasing levels of redistribution with both 25 μM ($p<0.001$) and 50 μM ($p<0.001$) showing significant percentage of redistributed cells over untreated conditions (Figure 3C). To our surprise, we found that NMDAR activation also rapidly induced GFP-Nedd4-1 redistribution into puncta (NMDA, 50μM for 3min) ($p<0.01$, n=150; **Fig. 3** A, B). This is completely blocked by the pretreatment of NMDAR antagonist, APV (10μM for 1 h),

(*p*=0.53, n=150; **Fig. 3** A, B). Some could argue that NMDA receptors activation indirectly activate AMPA receptors to then drive GFP-Nedd4-1 redistribution. To answer these questions, we pretreated cells with APV (50 μM for 1hr) before stimulating with AMPA (10 μM for 10 min) and saw no change in our redistribution phenotype as compared to AMPA treated cells (*p*=0.86, n=100; **Fig. 4**). We also pretreated cells with AMPA receptor antagonist, NBQX (50 μM for 1 h), before stimulating with NMDA (50 μM for 10 min) and saw no change in our redistribution phenotype as compared to NMDA treated cells (*p*=0.57, n=100; **Fig. 4**). Together these data indicate both AMPARs and NMDARs independently regulate the redistribution of GFP-Nedd4-1. Putting to rest the previous conundrum, the novel finding that NMDAR activation does in fact regulate the trafficking and redistribution of Nedd4-1 suggests that Nedd4-1 may be important for NMDAR signaling and plasticity.

The dynamic trafficking and distribution of GFP-Nedd4-1 in hippocampal neurons can be imaged in live time-lapse microscopy.

Previous studies using static imaging have shown us that activity mediated redistribution of Nedd4-1 occurs on a timescale of 5-10min (Scudder SL. et al, 2014). In this study, we used an attenuated Sindbis construct to virally express either GFP-Nedd4-1 or a control GFP cell-fill in primary hippocampal cultures (DIV 18-21). The cells were placed in our live imaging chamber at 37°C. Consistent confocal imaging parameters were used across experiments (See Materials and Methods). A 1 min video was taken to set a baseline for GFP and GFP-Nedd4-1 distribution. GFP-Nedd4-1 expressing cells were treated with H_2O (n=12) or AMPA (10 μM; n=10) at the 1 min time point and imaged for another 9 min. AMPA treated cells showed a significant increase in the average number of Nedd4-1 puncta starting

at 2 min and peaking around 5 - 7 min as compared to untreated cells ($p<0.01$; **Fig. 5** A, B). Nedd4-1 trafficking videos also show a diversity of activity with some puncta jittering back and forth, staying stationary, or participating in long range motion across the dendrite. GFP filled cells were also treated with AMPA (10 μM; n=7) and we saw no change in distribution as compared to H2O treated GFP-N41 expressing cells ($p=0.43$; **Fig. 5** A, B). This supports our claim that what we are observing is activity-dependent redistribution of an E3-ligase over time as opposed to an artifact of protein overexpression. This data suggests that we can confidently live image the trafficking dynamics of the E3-ligase Nedd4-1 in neurons.

AMPA and NMDA activation drive independent trafficking dynamics of Nedd4-1

Because we now have a method of observing Nedd4-1 live activity-dependent redistribution, we next investigated these trafficking dynamics under varying glutamatergic stimulations. Similar to our static experiments, we found that both AMPA and NMDA treatment drove the redistribution of Nedd4-1 over time. There was a significant increase in the Nedd4-1 average puncta count in AMPA and NMDA treated cells as compared to H_2O treated cells (respectively; $p<0.0001$, n=9; $p<0.001$, n=9; **Fig. 6** A-C). Interestingly, when using kymograph analysis to analyze Nedd4-1 puncta trafficking dynamics, we find that AMPA stimulation drives significantly different puncta formation and trafficking than NMDA stimulation (**Fig 7** A). Our data suggests that AMPA activation drives much more mobility of Nedd4-1 puncta than NMDA. AMPA stimulated cells have a lower latency to redistribution than NMDA stimulated cells suggesting that AMPA drives redistribution quicker in neurons (**Fig. 7** B). AMPA treated cells (n=452) showed a significantly higher average velocity of Nedd4-1 puncta mobility than NMDA treated cells (n=273)

(p<0.0001**Fig. 9**). We also observed a significant increase in size of Nedd4-1 puncta in AMPA treated (n=395) vs NMDA treated cells (n=220) (p<0.0001; **Fig. 8**). Finally, we observed a difference in the direction of puncta movement in AMPA and NMDA treated cells away from (anterograde) or towards the soma (retrograde). AMPA treated cells showed no significant difference in average anterograde or retrograde movement (respectively, n=371, n=374, p=0.07; **Fig 10**). In contrast, puncta in NMDA treated cells showed significantly higher average time spent moving towards distal dendrites (anterograde) than the soma (retrograde) (respectively, n=212, n=206, p<0.01; **Fig 10**). NMDA receptor stimulation seems to be driving Nedd4-1 puncta out towards distal dendrites and sequestering them at a specific location whereas AMPA stimulation seems to cluster much higher levels of Nedd4-1 protein to many more mobile sites. These results suggest that differing glutamatergic signals may engage Nedd4-1 in separate downstream pathways.

E. Discussion

In this study, we were able to successfully build a live imaging platform to record the trafficking dynamics of Nedd4-1 in neurons. We validated our activity dependent redistribution in our live imaging assay and used kymograph analysis to identify AMPAR and NMDAR specific regulation of Nedd4-1 subcellular localization.

Using live time-lapse imaging of GFP-Nedd4-1 we show that AMPAR and NMDAR activation leads to significantly different trafficking dynamics of the ligase. To our

knowledge, these are the first ever studies describing activity-dependent trafficking of a ubiquitin E3 ligase in neurons. By fluorescently labeling Nedd4-1 and taking advantage of the ligase's activity dependent recruitment to the synapse, we are able to track the movement of Nedd4-1 within the neuron over time. These findings nicely support our mass spec studies of the activity-dependent changes in the Nedd4-1 interactome in Chapter 4. We additionally observe that GFP-Nedd4-1 trafficking dynamics vary from AMPA or NMDA stimulation. NMDA treatment leads to smaller, slower moving puncta that spend more time moving towards distal dendrites. It is possible that NMDARs are recruiting and sequestering Nedd4-1 out in distal dendrites at the synapse. Unfortunately, our computational power limits the temporal resolution we can achieve in our live imaging (0.067 fps), likely causing us to miss some of the finer trafficking dynamics. Nevertheless, being able to study the trafficking dynamics of an E3 ligase in this manner is a huge step forward in uncovering Nedd4-1's role at in the neuron. In the future, this time-lapse technique can be paired with single synapse local activation to understand the rules regulating local recruitment, mRNA imaging techniques to probe at subcellular localization of Nedd4-1 translation (Hsia HE. et al. 2015), as well as many different synaptic plasticity paradigms to better define the fine regulation of Nedd4-1 trafficking in the neuron.

F. Acknowledgments

Chapter 3, in part, is currently being prepared for submission for publication of the material. Kevin White; Hoffner, Nicole, Gilmore, Stephen, Dozier, Lara; Patrick Gentry N. The dissertation author was the primary investigator and author of this material.

G. Figures and Tables

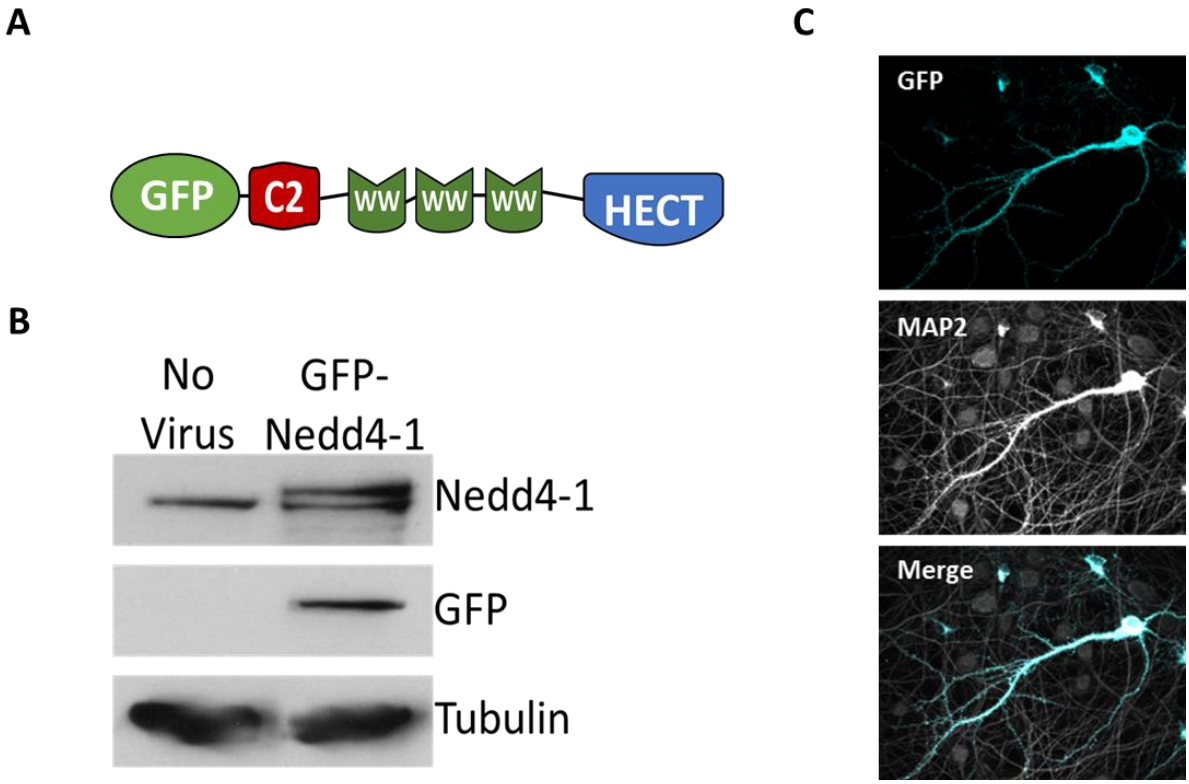

Figure 1 GFP-Nedd4-1 expression in hippocampal neurons. A) Schematic of GFP-tagged Nedd4-1 construct. B) Representative western blots of lysates from dissociated cortical neuron cultures infected with sindbis GFP-Nedd4-1. C) Immunofluorescent images of dissociated hippocampal neurons 18–21 DIV expressing GFP-tagged Nedd4-1 (Sindbis) for 16–20 h. Representative maximum z-projected confocal images.

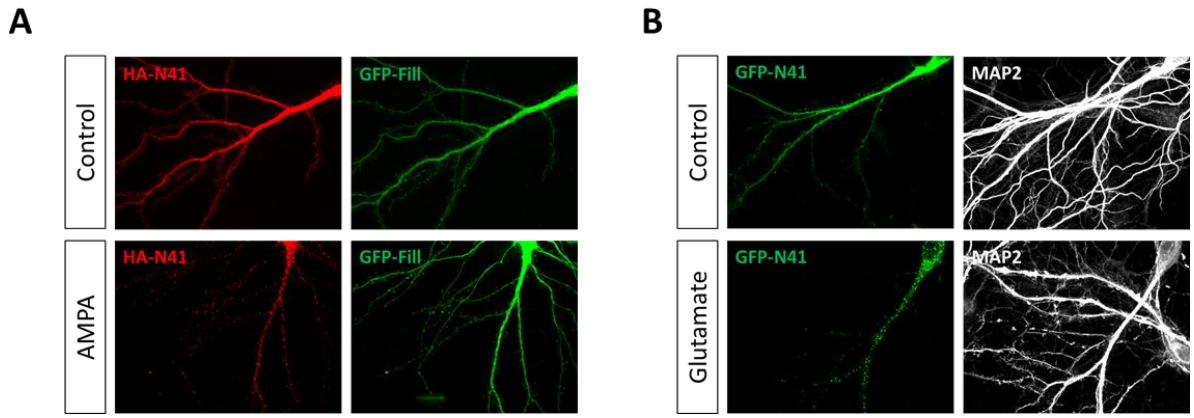

Figure 2 Comparison of HA-Nedd4-1 and GFP-Nedd4-1 expression and activity dependent redistribution

A) Representative immunofluorescent images of dissociated hippocampal neurons 18–21 DIV expressing HA-tagged Nedd4-1 (Sindbis) for 16–20 h either untreated or treated with AMPA 10 μM for 10 min. B) Representative images of GFP-tagged Nedd4-1 expressing cells untreated or treated with glutamate 100 μM and glycine 10 μM for 10 min.

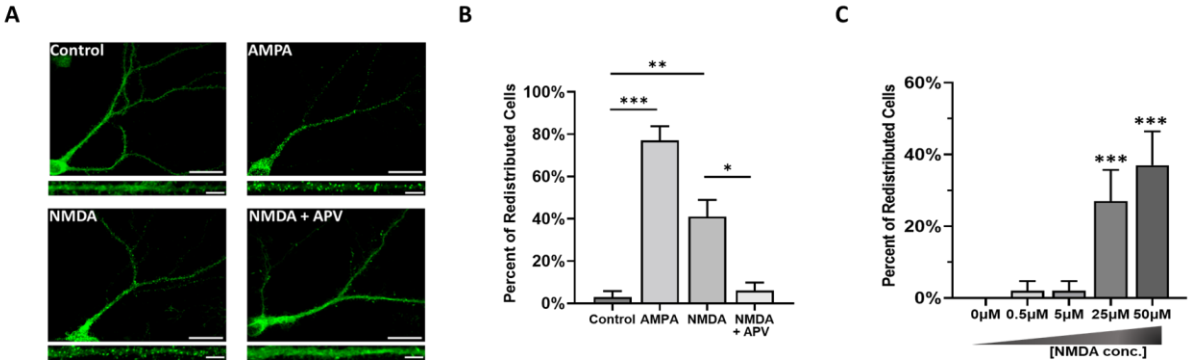

Figure 3 Stimulation with AMPA and NMDA lead to GFP-Nedd4-1 redistribution.
A) Representative immunofluorescent images of dissociated hippocampal neurons 18–21 DIV expressing GFP-tagged Nedd4-1 (Sindbis) for 16–20 h and treated with AMPA (10 μM) (n=150), treated with NMDA (50 μM) for 10min and washed at 3min (n=150), or pretreated with APV (50 μM) for 1hr and then treated with NMDA (50 μM) for 10min and washed at 3min (n=150), or left untreated (n=150). Maximum z-projected confocal images of whole cell and dendrite are depicted. B) Percentage of redistributed cells was quantified using visual analysis and the two-population proportional statistical test. C) Quantification of the percentage of redistributed cells with increasing concentration of NMDA treatment [0.5 μM, 5 μM, 25 μM, 50 μM] (n=100).

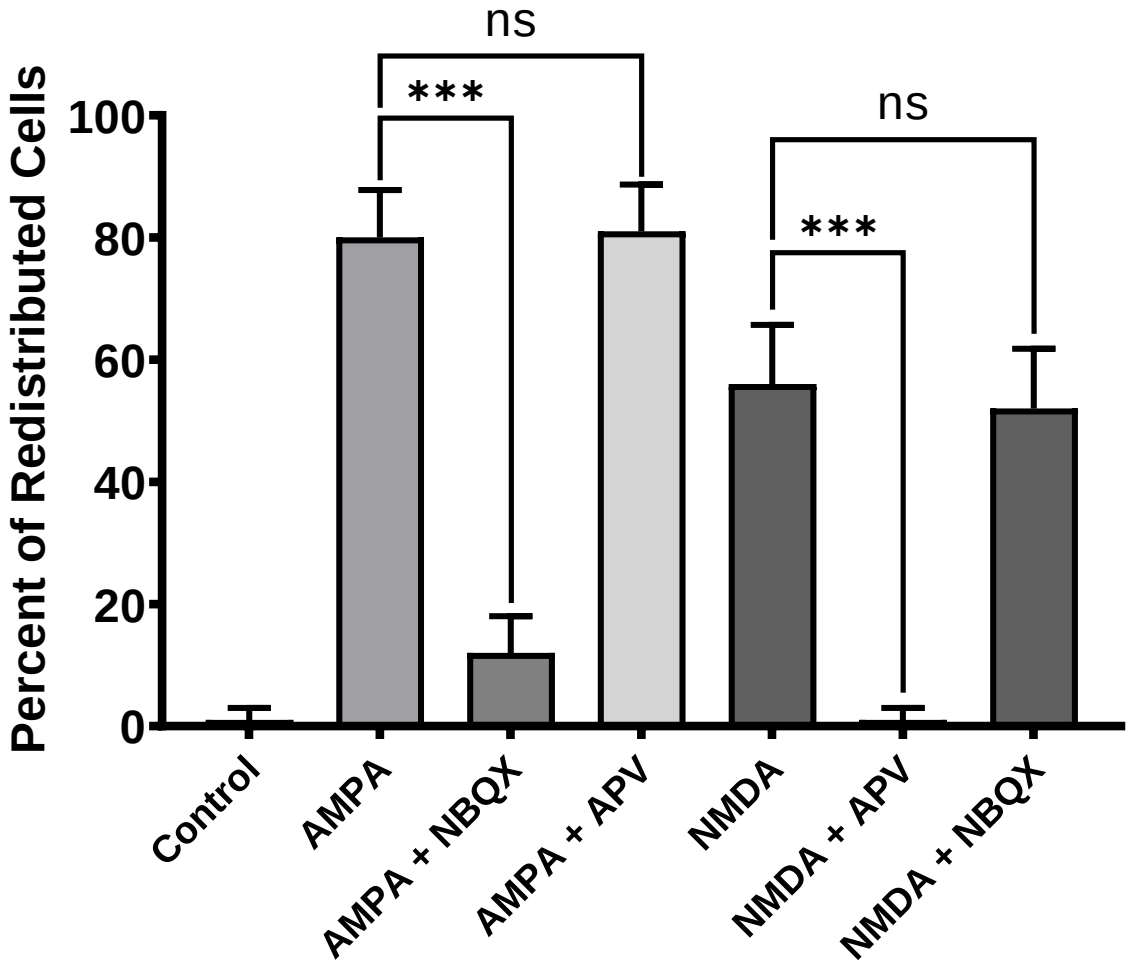

Figure 4 AMPA and NMDA independently stimulate redistribution of Nedd4-1.
In order to understand the indirect effects of AMPA and NMDA receptor activation on each other, dissociated hippocampal neurons 18–21 DIV expressing GFP-tagged Nedd4-1 (Sindbis) for 16–20 h were either left untreated, pre-treated for 1 h with the AMPA receptor inhibitor NBQX (50 μM), or pre-treated for 1hr with the NMDA receptor inhibitor APV (50 μM). Pre-treated cells were then treated at the end of 1 h with either AMPA (10 μM) or NMDA (50 μM) for 10min. Bar graph quantifies the percent of redistributed cells in each condition (n=100). Percentage of redistributed cells was quantified using visual analysis and the two-population proportional statistical test.

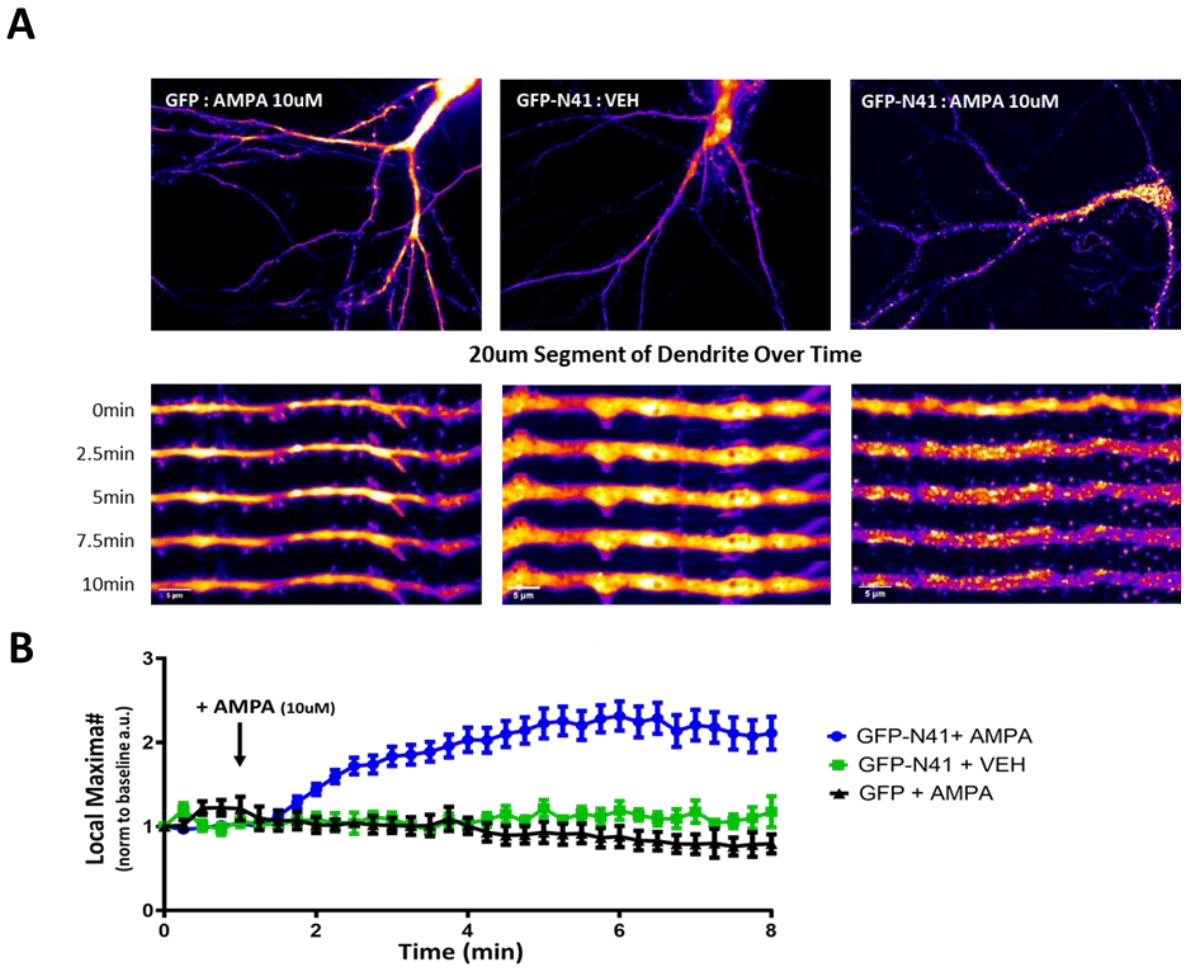

Figure 5 Stimulation with AMPA causes redistribution of Nedd4-1 in a live imaging context.

A) GFP-tagged Nedd4-1 or eGFP (Sindbis) were virally transduced in dissociated hippocampal neurons 18–21 DIV for 16–20 h. Cells were imaged on a spinning disk confocal microscope for 10 min acquiring images every 15 s. Representative images show neurons over a 10 min live imaging session expressing eGFP and treated with AMPA 10 µM at 1 min (n=7), GFP-N41 treated with H_2O at 1 min (n=12), or GFP-N41 treated with AMPA at 1 min (n=10). ImageJ image processing software was used to straight dendrites and analyze a 50 µm section 25 µm out from the cell body. B) Puncta count was quantified using an in-house local maxima macro and analyzed using One Way ANOVA statistical analysis.

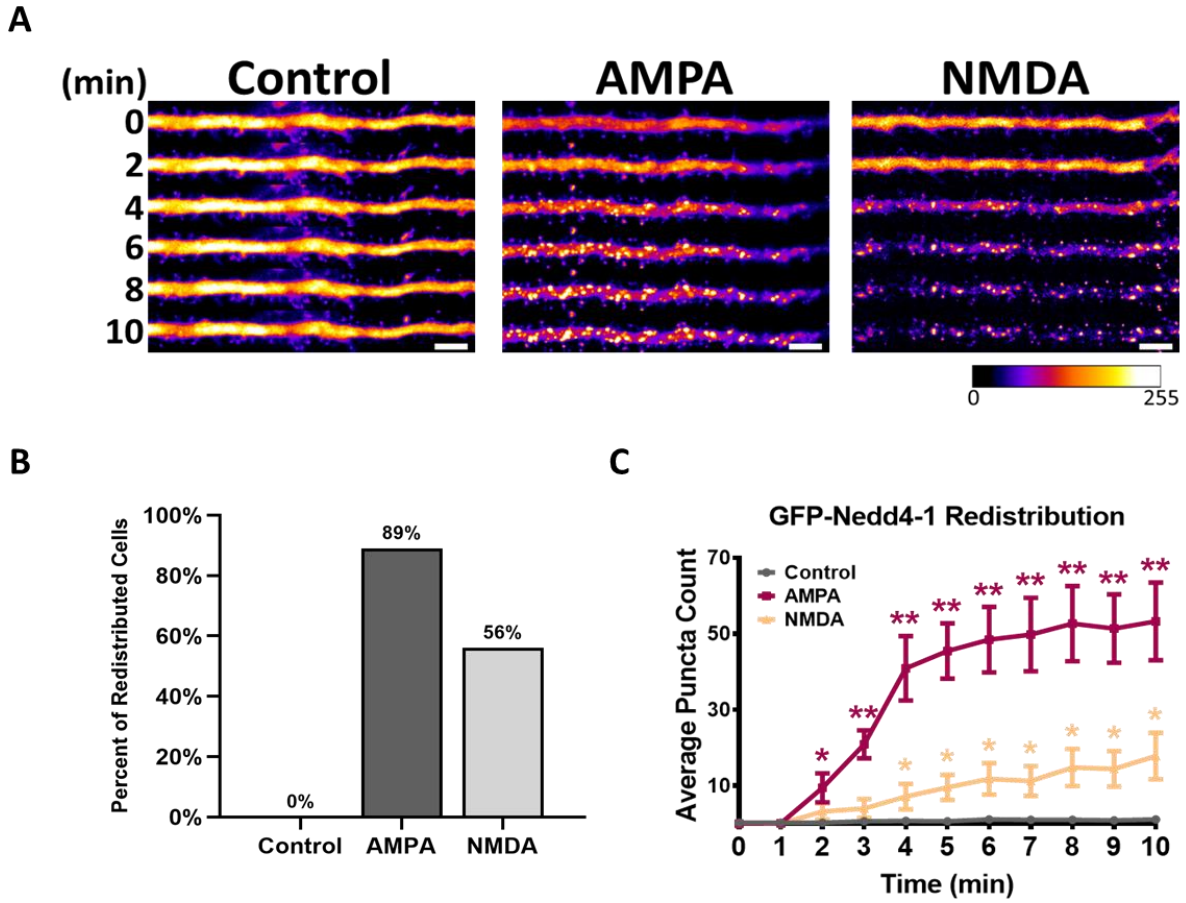

Figure 6 Stimulation with AMPA and NMDA drive GFP-Nedd4-1 redistribution in a live imaging context. A) GFP-tagged Nedd4-1 (Sindbis) was virally transduced in dissociated hippocampal neurons 18–21 DIV for 16–20 h. Cells were imaged on a spinning disk confocal microscope for 10 min acquiring images every 15 s. Representative images show neurons over a 10 min live imaging session treated with H_2O at 1 min (n=9), AMPA 10 µM at 1 min (n=9), NMDA 50 µM at 1 min (n=9). ImageJ image processing software was used to straight dendrites and analyze a 50 µm section 25 µm out from the cell body. B) Percentage of redistributed cells was quantified using visual analysis and two-population proportional statistical test. C) Average puncta count was quantified using visual analysis and One Way ANOVA statistical analysis.

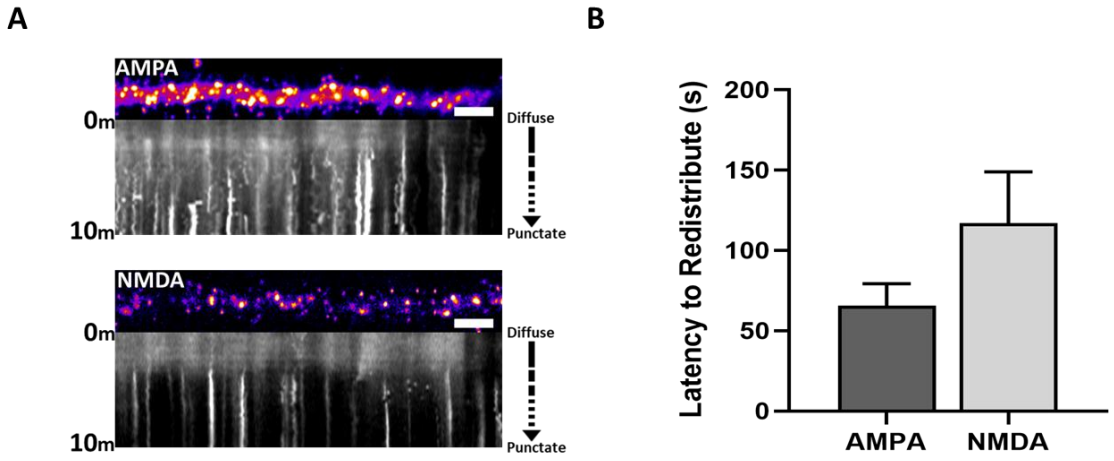

Figure 7 AMPA and NMDA induce distinct GFP-Nedd4-1 trafficking dynamics.
A) GFP-tagged Nedd4-1 (Sindbis) was virally transduced in dissociated hippocampal neurons 18–21 DIV for 16–20 h. Cells were imaged on a spinning disk confocal microscope for 10 min acquiring images every 15 s. Representative images show dendrites treated with AMPA 10 μM at 1 min (n=8) or NMDA 50 μM at 1 min (n=4). ImageJ was used to straighten dendrites and Kymographclear software was used to generate kymographs. Kymographdirect software was used to identify puncta and quantify their mobility. B) Visual analysis was used to quantify the start frame of puncta formation.

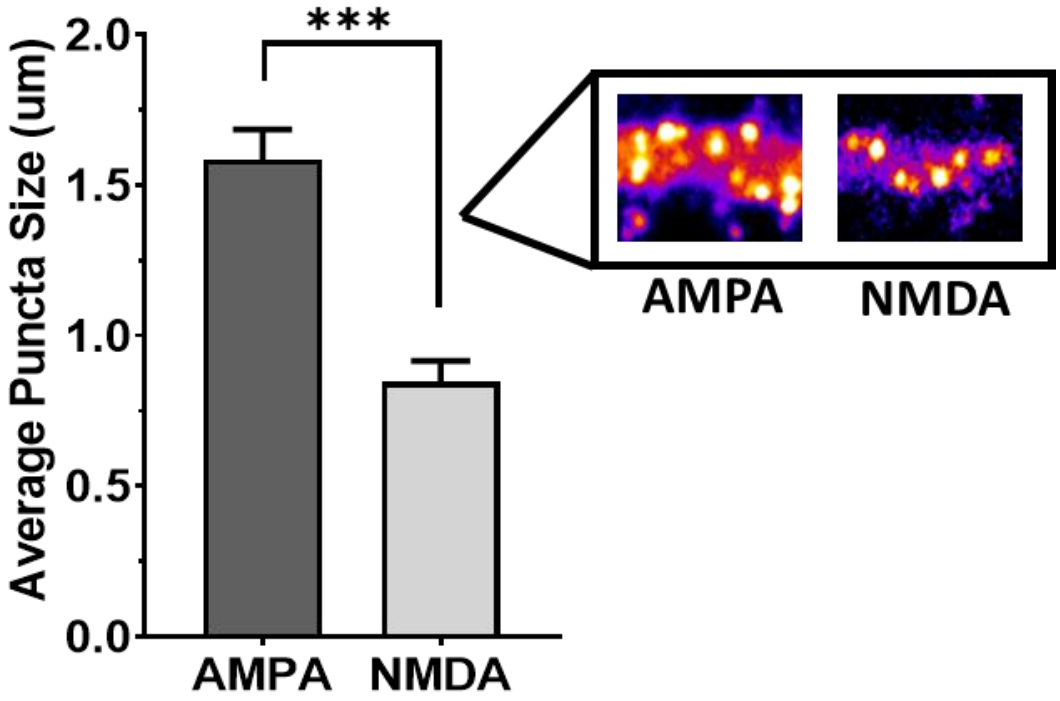

Figure 8 AMPA stimulation forms larger Nedd4-1 puncta.
Particle size was analyzed in AMPA (n=395) and NMDA (n=220) treated videos at the 10 min time point. ImageJ macro "Analyze Particles…" along with t-test statistical analysis was used to quantify a significant difference in puncta size.

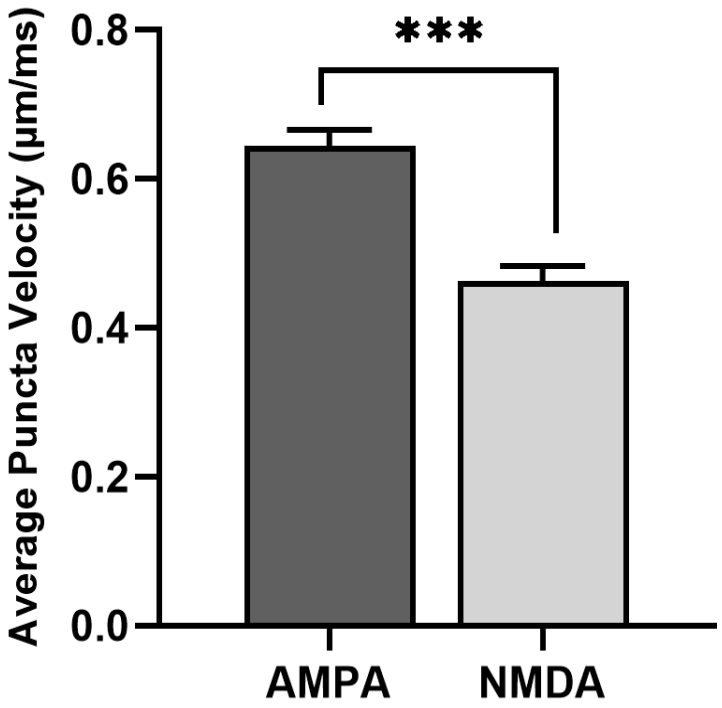

Figure 9 AMPA causes higher mobility in Nedd4-1 puncta.
Particle velocity was analyzed in AMPA (n=452) and NMDA (n=273) treated videos at the using KymographDirect to process KymographClear generated kymographs. Statistics were performed in Graphpad Prism using t-test statistical analysis.

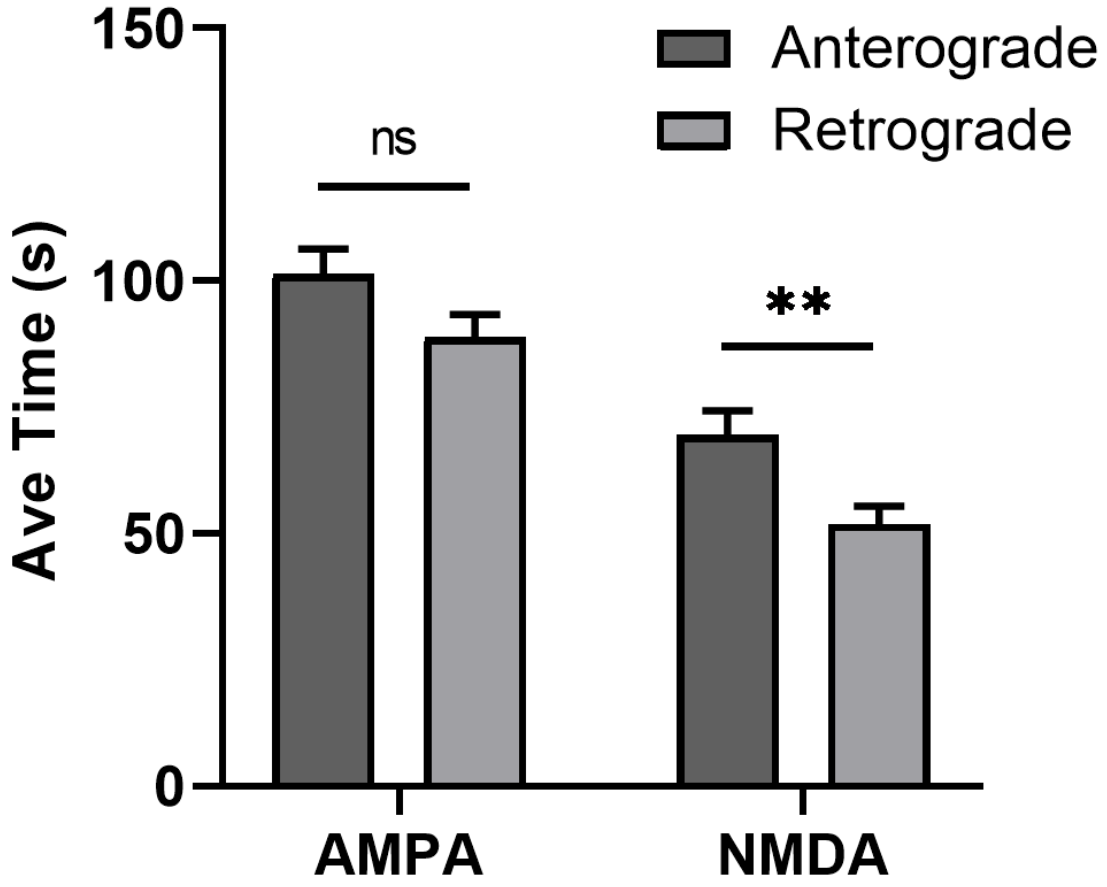

Figure 10 Puncta have increased anterograde movement in NMDA treated cells.
Direction of motion was analyzed in AMPA and NMDA treated videos at the using KymographDirect to process KymographClear generated kymographs. Nedd4-1 puncta in AMPA treated cells showed no difference in average time spent moving in anterograde (towards distal dendrites) (n=371) and retrograde (towards the cell body) (n=374) directions. On the other hand, NMDA showed an increase in average time spent moving anterograde (n=212) vs retrograde (n=206) direction. Statistics were performed in Graphpad Prism using t-test statistical analysis.

H. References

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Lin A, Hou Q, Jarzylo L, Amato S, Gilbert J, Shang F, Man HY. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. J Neurochem. 2011;119(1):27-39. doi:10.1111/j.1471-4159.2011.07221.x

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Djakovic SN, Schwarz LA, Barylko B, DeMartino GN, Patrick GN. Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. J Biol Chem. 2009;284(39):26655-26665. doi:10.1074/jbc.M109.021956

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Djakovic SN, Schwarz LA, Barylko B, DeMartino GN, Patrick GN. Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. J Biol Chem. 2009;284(39):26655-26665. doi:10.1074/jbc.M109.021956

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Djakovic SN, Marquez-Lona EM, Jakawich SK, Wright R, Chu C, Sutton MA, Patrick GN. Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. J Neurosci. 2012;32(15):5126-5131. doi:10.1523/JNEUROSCI.4427-11.2012

Djakovic SN, Marquez-Lona EM, Jakawich SK, Wright R, Chu C, Sutton MA, Patrick GN. Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. J Neurosci. 2012;32(15):5126-5131. doi:10.1523/JNEUROSCI.4427-11.2012

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Mangeol P, Prevo B, Peterman EJ. KymographClear and KymographDirect: two tools for the automated quantitative analysis of molecular and cellular dynamics using kymographs. Mol Biol Cell. 2016;27(12):1948-1957. doi:10.1091/mbc.E15-06-0404

Shepherd JD, Huganir RL. The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol. 2007;23:613-643. doi:10.1146/annurev.cellbio.23.090506.123516

Hsia HE, Kawabe H. Translational regulation of the expression of the ubiquitin E3 ligase Nedd4-1 by mTORC1-dependent signaling. Commun Integr Biol. 2015;8(4):e1017161. Published 2015 Aug 7. doi:10.1080/19420889.2015.1017161

Chapter IV

Activity-dependent changes in the Nedd4-1 interactome

A. Abstract

Protein homeostasis is the process of regulating the synthesis, maintenance and degradation of all proteins in the cell. Neuronal cells in particular depend on protein homeostasis to quickly and accurately change their proteomic composition to respond to external stimuli. E3 ligases are a family of proteins that contribute to protein homeostasis by ubiquitinating target proteins for degradation in the lysosome/proteasome. Although these proteins are well studied for their participation in protein homeostasis and subsequently neuronal cell health and function, much is still unknown about how they are regulated within the neuron and what biological pathways they may be involved in. In this study, we focus on a particular E3 ligase, Neuronal expressed downregulated in development protein 4-1 (Nedd4-1), that is known for playing a role in synapse plasticity, neurite outgrowth, and a number of important neuronal functions. To understand more about the pathways Nedd4-1 interacts with, we use APEX2-mediated proximity labeling to characterize the interactome of Nedd4-1.

First, we build an APEX2 tagged Nedd4-1 construct and validate its ability to biotin label Nedd4-1 interactors using mass spectrometry analysis. Next, we take advantage of Nedd4-1's activity dependent translocation in the neuron to characterize activity dependent changes in Nedd4-1's interactome. In untreated conditions, we find that Nedd4-1 interacts with spliceosomes and other RNA processing proteins. We also see that NMDA receptor activation drives Nedd4-1 to interact with synaptic organization proteins. Finally, we explore a functional role for Nedd4-1 at the synapse and find that blocking Nedd4-1 activity with inhibitor 1-benzyl-indole-3-carbinol (1B-I3C) ameliorates NMDA-induced reduction of GluA1 containing AMPARs. Together this evidence suggests that Nedd4-1 is involved in a

number of novel pathways previously undescribed, and that glutamatergic activation regulates Nedd4-1 interactions on a fine scale.

B. Introduction

The ubiquitin-proteasome system (UPS) is essential to protein homeostasis and cell health. Proteomic studies have described thousands of proteins with ubiquitination sites that are likely ubiquitinated at some point in their life cycle (Swatek KN. et al, 2016; Peng J. et al, 2003; Kim W. et al, 2011; Wagner SA. et al, 2011). This ubiquitination is mediated by a three-part system of E1 activating enzymes, E2 conjugating enzymes, and E3 ligating enzymes that bind ubiquitin molecules and attach them to lysine residues on their substrate targets. What is interesting about ubiquitin molecules is that their ability to be modified themselves greatly expands the diversity of downstream pathways they can target a substrate for. Single ubiquitin tags (monoubiquitination) can also themselves be ubiquitinated on their several lysine residues creating a chain of ubiquitination (polyubiquitination). Ubiquitin molecules on these chains can also be ubiquitinated in more than one place forming branched ubiquitin chain structures. On top of this, ubiquitin molecules can be modified with other post-translational modifications – phosphorylation, acetylation, SUMOylation. These variations of ubiquitin modification can be combined in unique ways that change their effect on downstream targets. This diversity is what makes the ubiquitin system so essential to cell function. Ubiquitination can regulate degradation in the lysosome/proteasome, mitophagy (Cunningham CN. et al, 2015), cell cycle control (Wickliffe KE. et al, 2011), DNA damage response (Gatti M. et al, 2015), immune signaling (Fiil BK. et al, 2013), epigenetic regulation

(Licchesi JD. et al, 2011; Jin J. et al, 2016), and protein trafficking (Yuan WC. et al, 2014). Understanding the "ubiquitin code" and the hierarchy of ubiquitin signaling and modifications will give us a greater understanding of cell function entirely, especially in the CNS.

For this reason, many labs study E3 ubiquitin ligases and their various roles within the neuron. One way of studying these ligases is by characterizing the network of proteins they interact with (interactome). Some labs use techniques like fluorescent co-localization and biochemical co-immunoprecipitation to define novel interactors. The drawback to these techniques is that they require you to already have an idea of what proteins the E3 ligase may be interacting with. That is why many other labs take a more unbiased approach by pairing immunoprecipitation with mass spectrometry analysis to characterize the interactome of proteins associating with their ligase of interest. Numerous labs have used this technique to study various E3 ligases and their role in the central nervous system (CNS) (Yalcin E. et al. 2019; Ding X. et al. 2020; Zhu J. et al. 2019; Dong Z. et al. 2020). Although a lot can be learned using this technique, co-immunoprecipitation will not identify any transient interactions with your protein of interest. Interactors with your protein of interest must make a strong enough bond to survive bead purification and washing to be identified in this method. Because of this some scientists have begun using proximity labeling assays to define protein interactomes. These methods use an enzyme to label any protein within a tight spatial range which increases sensitivity of labeling and captures even transient interactions. E3 ligases are only recently being studied this way with a majority those studies occurring within the last year (Howley BV. et al. 2022; Zhai Y. et al. 2022; Cao X. et al. 2022; Zuzow N. et al. 2018; Coyaud E. et al. 2015) and none of them in the CNS.

In this study, we set out to use proximity labeling to define the Nedd4-1 interactome in dissociated hippocampal neurons. Nedd4-1 is a well-studied E3 ligase known to play a crucial role in neuronal health and function. Nedd4-1 activity is implicated in regulation of receptor trafficking (Schwarz LA. et al, 2010; Scudder SL. et al, 2014; Lin A. et al, 2011; Hou Q. et al, 2011; Scudder SL. et al, 2014; Wei J. et al, 2016; Gautam V. et al, 2013; Lee S. et al, 2019; Persaud A. et al, 2011), axonal branching and outgrowth (Kawabe H. et al, 2010; Fu Z. et al, 2007; Taira K. et al, 2004; Christie KJ. et al, 2012; Hsia HE. et al, 2014), gap junction formation (Giaume C. et al, 2010; Liao CK. et al, 2013), and many other biological pathways. Homozygous knockout of Nedd4-1 is embryonically lethal and even heterozygous knock out of Nedd4-1 in mice show mobility and gait abnormalities (Liu Y. et al, 2009; Camera D. et al, 2014). Nedd4-1 has also been described to play a role in the pathophysiology of dementia (Kwak Y. et al, 2012; Rodrigues EM. et al, 2016; Guntupalli S. et al, 2017; Zhang Y. et al, 2018; Tofaris GK. et al, 2011; Sugeno N. et al, 2014; Mund T. et el, 2018; Won SY. et al, 2022), psychiatric disorders (Warnica W. et al, 2015; Han C. et al, 2019), cancer (Dai B. et al, 2010; Chuang HY. et al, 2021; Rimkus TK. et al, 2022), and other neurological disorders. This evidence highlights the importance of Nedd4-1 in the CNS. Characterizing Nedd4-1's interactome will add to our overall understanding of how this ligase functions in neurons.

C. Materials and Methods

Antibodies and reagents. Antibodies were as follows: pAb GFP (Invitrogen), pAb MAP2 (Abcam), mAb Nedd4-1 (Biosciences), mAb PSD-95 (EMD Millipore), Strepavidin-HRP

(BioLegend). Reagents were as follows: AMPA, NMDA, Biotin-phenol, hydrogen peroxide, Trolox, Sodium L-ascorbate, NHS-LC-Biotin (Peirce), neutravidin-agarose (Pierce).

Sindbis constructs. The sindbis APEX2-HA-Nedd4-1; GFP construct was created by cloning the APEX2 sequence from a hPGK-NES-APEX2-GFP construct (provided by the Yeo Lab) into a 2 gene sindbis HA-Nedd4-1; GFP construct (in lab). Production of recombinant sindbis virus was performed as described previously (Djakovic et al., 2009). All DNA and viral constructs were verified by sequencing.

Neuron cultures and infections. Rat dissociated hippocampal or cortical neurons from postnatal day 1 pups of either sex were plated at a density of 45,000 cells/cm2 onto poly-d-lysine-coated coverslips, poly-d-lysine-coated 6-well plastic dishes at ~500,000 cells per well (cortical cultures), or poly-d-lysine-coated 10cm plastic dishes at ~3,000,000 cells dish (cortical cultures). These cultures were maintained in B27 supplemented neurobasal medium (Invitrogen) until ≥18 d in vitro (DIV), as described previously (Scudder et al. 2014, Djakovic et al., 2009; Schwarz et al., 2010; Djakovic et al., 2012). Hippocampal or cortical cultures were infected with sindbis virion at DIV 18–21 and allowed to express for 16–20 h.

Immunostaining. After infections and drug treatments, neurons were washed with cold PBS-MC and fixed with a solution containing 4% paraformaldehyde and 4% sucrose for 10 min. Cells were then permeabilized with 0.2% Triton X-100 and 2% BSA in PBS-MC for 20 min, followed by a 1 h block in 5% BSA in PBS-MC. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons for 12-20 h at 4°C or 1 h at room temperature respectively. Coverslips were mounted onto glass slides for confocal imaging.

Western Blots. 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 incubating for 20 min at 4°C. Protein concentration was determined by BCA protein assay (Pierce) and equal protein amounts were loaded. Samples were boiled with sample buffer, resolved on 8% SDS page, and probed with primary antibodies. Blots were digitized and band intensities quantitated using ImageJ. For quantification target protein levels after treatment of neuronal cultures, band intensities in each condition were normalized to tubulin band mean intensity from the same sample.

Co-Immunoprecipitations. Cultured rat cortical neurons were lysed in buffer (50 mm Tric-HCl, 150 mm NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) with protease inhibitors. Homogenates were cleared by centrifugation at 14,000 rpm at 4°C. For immunoprecipitations (IPs), cleared lysates were incubated with primary antibodies at 4°C overnight, after which protein A or protein G agarose beads were added for an additional 1 h (Pierce). Immunoprecipitates were then washed, boiled in sample buffer, resolved on SDS PAGE, and probed in Western blot analysis.

APEX2 mediated biotinylation. 10cm dishes of cultured cortical rat neurons were used at DIV 18-21. 500μM biotin-phenol (BP) was added to the medium for 1hr at 37C. APEX labeling was performed by adding hydrogen peroxide to a final concentration of 1mM for 60 s before quenching the biotinylation reaction by adding Trolox ((+/−)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma 238813) and sodium L-ascorbate (Sigma A4034) to a final concentration of 5 and 10mM, respectively.

Sample prep. Cells were lysed in 8M urea, 50 mM ammonium bicarbonate (ABC) and benzonase and the lysate was centrifuged at 14,000 x g for 15 minutes to remove cellular debris. Supernatant protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Thermo Scientific). Disulfide bridges were reduced with 5 mM tris(2-carboxyethyl) phosphine (TCEP) at 30°C for 60 min, and cysteines were subsequently alkylated with 15 mM iodoacetamide (IAA) in the dark at room temperature for 30 min.

Affinity purification. Affinity purification was carried out in a Bravo AssayMap platform (Agilent) using AssayMap streptavidin cartridges (Agilent). Briefly, cartridges were first primed with 50 mM ammonium bicarbonate, and then proteins were slowly loaded onto the streptavidin cartridge. Background contamination was removed with 8M urea, 50 mM ammonium bicarbonate. Finally, cartridges were washed with Rapid digestion buffer (Promega, Rapid digestion buffer kit) and proteins were subjected to on-cartridge digestion with mass spec grade Trypsin/Lys-C Rapid digestion enzyme (Promega, Madison, WI) at 70C for 1h. Digested peptides were then desalted in the Bravo platform using AssayMap C18 cartridges and dried down in a SpeedVac concentrator.

Mass spectrometry analysis. Prior to LC-MS/MS analysis, dried biotin-enriched peptides were reconstituted with 2% ACN, 0.1% FA and concentration was determined using a NanoDropTM spectrophometer (ThermoFisher). Samples were then analyzed by LC-MS/MS using a Proxeon EASY-nanoLC system (ThermoFisher) coupled to a Orbitrap Fusion Lumos Tribid mass spectrometer (Thermo Fisher Scientific). Peptides were separated using an analytical C18 Aurora column (75µm x 250 mm, 1.6 µm particles; IonOpticks) at a flow rate of 300 nL/min (60C) using a 75-min gradient: 2% to 6% B in 1 min, 6% to 23% B in 45 min,

23% to 34% B in 28 min, and 34% to 48% B in 1 min (A= FA 0.1%; B=80% ACN: 0.1% FA). The mass spectrometer was operated in positive data-dependent acquisition mode. MS1 spectra were measured in the Orbitrap in a mass-to-charge (m/z) of 375 – 1500 with a resolution of 60,000. Automatic gain control target was set to 4 x 10^5 with a maximum injection time of 50 ms. The instrument was set to run in top speed mode with 1-second cycles for the survey and the MS/MS scans. After a survey scan, the most abundant precursors (with charge state between +2 and +7) were isolated in the quadrupole with an isolation window of 0.7 m/z and fragmented with HCD at 30% normalized collision energy. Fragmented precursors were detected in the ion trap as rapid scan mode with automatic gain control target set to 1 x 10^4 and a maximum injection time set at 35 ms. The dynamic exclusion was set to 20 seconds with a 10-ppm mass tolerance around the precursor.

Data analysis. All mass spectra from were analyzed with MaxQuant software version 1.6.11.0. MS/MS spectra were searched against the Rattus norvegicus Uniprot protein sequence database (downloaded in Jan 2020) and GPM cRAP sequences (commonly known protein contaminants). Precursor mass tolerance was set to 20ppm and 4.5ppm for the first search where initial mass recalibration was completed and for the main search, respectively. Product ions were searched with a mass tolerance of 0.5 Da. The maximum precursor ion charge state used for searching was 7. Enzyme was set to trypsin in a specific mode and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%. Statistical analysis and figure visualizations of differentially expressed proteins were conducted in R

using Differential Enrichment analysis of Proteomics data (DEP, version 1.22.0; Zhang et al, 2018).

Pathway Enrichment. For each given gene list, Metascape's (Zhou Y. et al, 2019) pathway and process enrichment analysis is carried out with the following ontology sources: GO Biological Processes, KEGG Pathway, Reactome Gene Sets, CORUM, and WikiPathways. All genes in the genome have been used as the enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the cumulative hypergeometric distribution2, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testings3. Kappa scores4 are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.

Electrophysiological recordings (Dore Lab/UC San Diego). Whole-cell recordings were obtained with Axopatch-1D amplifiers (Molecular Devices) using 3 to 5 MΩ pipettes with an internal solution containing (in mM) 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2ATP, 0.4 Na3GTP, 10 sodium phosphocreatine (Sigma), and 0.6 EGTA (Amresco), at pH 7.25. Spontaneous miniature excitatory postsynaptic currents (mEPSCs) in 18-21DIV primary hippocampal neurons held at −60mV. Primary hippocampal neurons were pre- treated overnight (16-18h) with either vehicle (DMSO), 20 μM bicuculline (Tocris), 40 μM 1-Benzyl-I3C (ChemDiv) or 20 μM bicuculline plus 40 μM 1-Benzyl-I3C. External

perfusion consisted of HBSS based solution containing: 0.87x HBSS, 5mM HEPES, 1mM Glucose, 1mM MgCl2, 1mM CaCl2, 0.4 μM 2-chloroadenosine, and 10 μM gabazine (Tocris) and 1uM tetrodotoxin (Tocris) (pH 7.4). mEPSCs were recorded for 1-10min and events were analyzed manually using the MiniAnalysis program (Synapto-soft) blind to experimental conditions; event threshold was set at 6-8pA.

F. Results

APEX2 mediated proximity labeling can be used to characterize activity dependent changes in the Nedd4-1 interactome

In this study, we utilize Nedd4-1's ability to translocate within the cell in an activity dependent manner to identify novel regulators and/or targets of Nedd4-1 redistribution. In order to do this, we needed an assay to tag proteins possibly interacting with Nedd4-1. The APEX molecule was developed in the Ting lab as an enzyme that cleaves inactive biotin phenol (BP) into active biotin that ubiquitously labels any protein in close enough spatial range (Hung V. et al, 2016). The fact that APEX's catalytic activity is only active in the presence of hydrogen peroxide (H_2O_2) allows you to limit the enzymes active time to a short time window. This temporal restriction only allows APEX enough time to cleave enough BP to label proteins within a 10-20nm radius and thus creates the spatial resolution of APEX mediated proximity labeling. In this study we will use the APEX2 enzyme which functions the same as its predecessor but with increased activity and sensitivity (Mair A. et al, 2022). We fused the APEX2 enzyme to our (attenuated) HA-tagged Nedd4-1 (**Fig 1** A). This will allow us to proximity label the Nedd4-1 interactome before and after activity dependent

translocation of the ligase. Once biotinylated, these interactors can be purified and identified using mass spectrometry analysis (**Fig 1** B).

In order to validate our APEX2-HA-Nedd4-1 construct, we virally expressed it in dissociated cortical and hippocampal cultures (DIV 18-21) (**Fig 1** C). Cultures with and without APEX2-HA-Nedd4-1 were introduced to BP (1 h), H_2O_2 (60 sec), or BP and H_2O_2. Using Immunocytochemistry and biochemical methods and probing with Streptavidin-HRP or Streptavidin-568, we see that only cells expressing APEX2-HA-Nedd4-1 that were treated with both BP and H_2O_2 showed biotin labeling of proteins (**Fig 2** A, B). Next, we wanted to confirm two important aspects of APEX2-HA-Nedd4-1. Because the translocation of Nedd4-1 is so important to the question we are trying to ask, we wanted to confirm that the addition of APEX2, which is approximately the same size as GFP (~27 kDa) (Lam SS. et al, 2015), does not affect the redistribution of Nedd4-1. We were able confirm this by treated proximity labeled APEX2-HA-Nedd4-1 expressing cells with AMPA (10 μM for 10 min) (**Fig 2** B). Here we see redistribution phenotypes comparable to what has been seen in our trafficking experiments and previous studies. Second, we wanted to confirm the spatial resolution of APEX2. It is important to our question that we are able to proximity label proteins in close spatial region of Nedd4-1 if we are to see any effects of translocation on the Nedd4-1 interactome. We straightened dendritic segments from AMPA treated and proximity labeled cells and found tight colocalization of APEX2-HA-Nedd4-1 signal and biotin signal (**Fig 2** C). Together these results gave us confidence in our ability to label activity dependent differences the Nedd4-1 interactome.

Now that we can confidently label the Nedd4-1 interactome, we continued on to confirm that biotin labeled proteins could be identified through mass spec analysis. Biotinylated proteins were purified and isolated from proximity labeled cultures treated with AMPA (10 μM for 10 min), NMDA (50 μM for 10 min), or left untreated. We expect that due to the differences found in the trafficking dynamics of AMPA and NMDA stimulated cells we would observe a similar difference in the proteomic profiles of these two stimulations. Samples from each condition were delivered to a mass spectrometry core where they performed LC-MS/MS analysis and data processing (See Materials & Methods). We are able to see here that not only does APEX2 proximity labeling enrich for biotinylated proteins (**Fig 3**), but when utilize hierarchical clustering and we analyze the top 500 variable proteins through PCA analysis we see a clustering of activity stimulated conditions (**Fig 4 & 5**). These data together suggest that we can both confidently label the Nedd4-1 interactome and identify activity dependent changes in the interactome.

Glutamatergic stimulation drives differences in the Nedd4-1 interactome

For any given protein the protein abundance in either of our three conditions control, AMPA, and NMDA were compared against each other. Any comparison with an adjusted p-value, false discovery rate (FDR) score, of less than 0.1 constituted that protein as an enriched hit (EH) for that condition. We found a total of 70 EHs out of a total of 2,510 mass spec hits: 45 in control, 8 in AMPA, and 23 in NMDA (**Fig 6** A, E). A majority of our EHs were found in the comparison of control and NMDA (**Fig 6** B-D). This was due to the low number of hits enriched in the AMPA condition. This likely had to do with the amount of variability in the AMPA samples and could be eradicated with a higher sample size. It is also important to note

that even though only 1 of our EHs showed any known interactions with Nedd4-1 in the literature, analysis of the amino acid sequence of all 70 EHs showed at least 10% these hits having a PPxY motif (**Table 1**). PPxY motifs are proline rich regions in a protein sequence that serve as a known binding site for the WW domains of Nedd4-1. This shows that a significant number of our EHs have a high probability of interacting with Nedd4-1.

To further characterize our Nedd4-1 interactomes, we used the online bioinformatics tool, Metascape. Metascape accepts a list of gene IDs that the site then runs through its pathway enrichment pipeline (See Materials & Methods) to identify significantly enriched biological and metabolic pathways that align with that list of genes. Because AMPA had so few EHs, we did not see any significantly enriched pathways involving a minimum of 3 EHs. On the other hand, control and NMDA showed crucial differences in their enriched pathways. Untreated control cells enriched for RNA processing and export pathways like mRNA splicing, splicing-associated factors complex, spliceosome complex assembly, positive regulation of mRNA splicing, regulation of alternative mRNA splicing via spliceosome, Eukaryotic translation and elongation, mRNA splicing – minor pathway, nucleic acid transport, membrane trafficking (**Fig 7** A; **Fig 8**). NMDA treated cells enriched for synaptic structure and dendritic growth pathways like regulation of postsynaptic organization, regulation of actin-filment based process, protein localization to the membrane (**Fig 7** B; **Fig 8**). This data together suggests that glutamatergic stimulation drives Nedd4-1 to associated with different biological pathways.

NMDA drives Nedd4-1 to associate with synapses

To probe deeper into the differences between contorl and NMDA interactomes we utilized a the synaptic datbase SynGo (Koopmans F. et al, 2019) to identify syanpse associated proteins in amongst our EHs. Eventhough both control and NMDA showed 7 synapse associated hits, these hits constitute 16% of total control EHs and 27% of total NMDA EHs. NMDA seems to drive a higher association with syanptic protiens (**Fig 9** A). There is also specificity in the types of synaptic associations in stimulated and unstimulated cells. Nedd4-1 in unstimulated conditions associated more strongly with synaptic vessicle fusion (*NAPA*, *RABGEF1*, *VAMP4*), ribosomal proteins (*RPL32*) and intracelular signaling (*PLCB1*) (**Fig 9** B). Whereas Nedd4-1 in NMDA stimulated cells associated specifically with transmembrane synaptic ancoring proteins (*ILRAPL1* and *NRCAM*), cytosolic synaptic structral proteins (*CASKIN1*, *ARHGAP44*, *BAIAP2*, *SHANK3*), and neruonal signaling molecules (*NOS1*) (**Fig 9** B). We supported these findings using immoncytochemistry in cultures expressing GFP-N41 and treated with AMPA (10 μM for 10min), NMDA (50 μM for 10min) or left untreated. We probed for synpatic marker, PSD-95, and used an ImageJ macro to analyze the person's coefficient (PC) to quantify the colocalization of Nedd4-1 puncta at the synapse. NMDA (n=17) stimulation drove Nedd4-1 to colocalize with PSD-95 significantly more than AMPA (n=27) stimulation ($p<0.001$; **Fig 9** C, D). Together these resuts suggest that NMDA recruits Nedd4-1 to associate with synaptic organization proteins.

Nedd4-1 is necessary for NMDA dependent changes in synaptic function

Now that we have shown that Nedd4-1 can be recruited to the synapse, the question remains what Nedd4-1's functional role is at the synapse. In order to answer this question we will use a small molecule called 1-benzyl-I3C that acts as antagonist for Nedd4-1 activity

(**Fig 10** A-C) (Quirit JG. et al, 2017). This drug is perfect for our purposes seeing as on top of block Nedd4-1 activity, 1B-I3C preserves the health of our neuronal cultures. Pretreatment with this inhibitor showed significant increase in inter-event interval (IEI) ($p<0.01$) and an partial attenuation of biccuculine induced increase in mini excitatory post-synaptic currents (mEPSCs) (**Fig 12** A, B). This re-enforces claims towards Nedd4-1's importance to synaptic function. Furthmore, we used biochemical assays to show that Nedd4-1 is not only involved but crucial to NMDA induced reduction in GluA1 receptors. NMDA depedent decrease in surface level GluA1 subunits was blocked by pretreatment of 1B-I3C (**Fig 13** A, B). This results was not seen with GluA2 subunits. It has been known that Nedd4-1 plays a major role in AMPA receptor trafficking but never in an NMDA receptor dependent manner. Together these results suggest a functional role for NMDA receptor recruitment of Nedd4-1 to the synapse.

Nedd4-1's role in neruological disease

In addition to our proteomic data bringing better understanding of Nedd4-1's role in healthy neurons, this data also shed so light on Nedd4-1's futher possible role in neruological disease. Nedd4-1 has a healthy body of literature indicating its function and/or dysfunction playing a crucial part in numerous neurological disorders. Now that we have defined the Nedd4-1 interactome, we used an in depth literature search of these novel interactors and characterize their association with certain neurological disorders. We recorded protein interactors with moderate to high (5 or more primary research articles) disease relevance (**Table 1**). 58.6% (41/70) of all EHs showed some type of disease relevance. This disease relevence changed depeding on the condition: 57.8% (26/45) of Control, 50% (4/8) of AMPA,

and 65.2% (15/23) of NMDA EHs. We focused our literature search on 9 different neurological disorders: Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), Multiple Sclerosis (MS), Glioma, Autism Spectrum Disorder (ASD), Schizophrenia (SZ), or Major Depressive Disorder (MDD). We also quantify what proportion of our three (control, AMPA, NMDA) interactomes makeup the EHs of each of these disease states (**Fig 14**). This comprehensive analysis will serve as a resource for other labs and future experiments when exploring Nedd4-1 interactions and possible involvement in the pathophysiology of these neurological disorders.

E. Discussion

In this study, we were able to successfully characterize activity dependent changes in the Nedd4-1 interactome. A few labs have, through a variety of methods, studied the interactome of Nedd4-1 (Yalcin E. et al. 2019; Hatstat AK. et al. 2021; Persuad A. 2009; Manning J. et al. 2020). Lohraseb I. et al. 2022 even characterized novel targets in neural crest cells. Our study is the first to use proximity labeling to successfully characterize the Nedd4-1 interactome in the CNS. APEX2-mediated proximity labeling identifies three separate interactomes: control, AMPA treated, and NMDA treated. Nedd4-1 associates with RNA processing proteins in untreated conditions while NMDA treatment drives increased synaptic enrichment and colocalization with postsynaptic markers. Pharmacological perturbation confirms that NMDAR recruitment of Nedd4-1 to synapses is necessary for NMDA-dependent downregulation of surface GluA1 containing AMPARs. Together these results

suggest that Nedd4-1 is involved in a number of novel pathways previously undescribed, and that glutamatergic activation regulates Nedd4-1 interactions on a fine scale.

Our first objective was to identify activity dependent changes in the Nedd4-1 interactome. Previous studies out of our lab have shown that Nedd4-1 aggregates and redistributes within the neuron in an activity dependent manner (Scudder SL. et al. 2014). The ligase's calcium binding (C2) domain allows it not only to bind to Ca^{2+} but to phospholipid membranes as well and translocate within the cell (Plant PJ. et al. 1997; Plant PJ. et al. 2000). Without this C2 domain Nedd4-1 cannot redistribute in response to neuronal activity (Scudder SL. et al. 2014). This unique characteristic of Nedd4-1 combined with the spatial resolution of our APEX2 mediated proximity labeling assay is what allows us to define activity dependent differences in the Nedd4-1 interactome. We used LC-MS/MS to analyze our samples which is notorious for high noise and non-specific binding. For this reason, we included a control condition not expressing APEX2 to validate the effectiveness of our proximity labeling. Conditions virally expressing our APEX2-HA-Nedd4-1 construct isolated a significantly higher number of proteins than in our uninfected control. This along biochemical and immunofluorescent experiments validating the efficacy of the proximity labeling assay gave us confidence in our ability to label Nedd4-1's interactome. When then validate that we can identify activity dependent changes in Nedd4-1's interactome using principal component analysis (PCA) and hierarchical clustering. Untreated, AMPA treated, and NMDA treated conditions showed significant differences in their interactome through clustering and comparison of enriched protein hits. Together these data validate our original hypothesis. One drawback to our experiments was that we identified very few enriched hits in our AMPA

treated condition. It is possible that this is because AMPA treatment drives Nedd4-1 to interact with fewer proteins. Although this might be true, we suspect that the low enrichment is due to a variability in our AMPA samples. AMPA treated cells did not show a decrease in the total number of isolated proteins, but it did show fairly low p-values when comparing protein abundances to other conditions. PCA clustering also indicated increased variability in AMPA conditions. Increased sample size would likely allow us to better describe AMPA induced changes in the Nedd4-1 interactome. Future experiments would take advantage of the temporal element of activity-dependent redistribution of Nedd4-1. Studies out of our lab show that Nedd4-1 redistribution takes place over the span of 4 - 6 min. Proximity labeling could be performed over various time points during the redistribution of Nedd4-1 to give us a fine temporal resolution of Nedd4-1's changing interactome. This would allow us to better understand Nedd4-1's role in the neuron and the pathways it interacts with.

Next, we successfully used bioinformatic techniques to identify novel biological pathways that Nedd4-1 associates with. Unexpectedly, we find that Nedd4-1 interacts with RNA processing pathways, specifically spliceosome complex components, in untreated conditions. There is very little literature describing a role for E3 ligases in RNA processing (Cano F. et al. 2010; Choudhury NR. et al. 2014), let alone interacting with spliceosomes complexes (Lokireddy S. et al. 2014; Swenson SA. et al. 2020). Nedd4-1 has been implicated in the regulation of an AID-associated RNA polymerase II (Sun J. et al. 2013) and a Ribonucleoprotein complex, Thoc1, (Song F. et al. 2013) but our study is the first to suggest a role for Nedd4-1 regulating spliceosome complexes. Nedd4-1 is often studied on the degradation side of protein homeostasis, but this work suggests a role for Nedd4-1 in the

regulation of protein synthesis as well. Some of the limitations to this study come from the use of APEX2 proximity labeling. The trade-off for the sensitivity to label transiently interacting proteins is an inability to confirm a physical interaction between Nedd4-1 and novel interactors. Future experiments will use co-immunoprecipitation to confirm a physical interaction between Nedd4-1 and interactome hits. This type of biochemical technique could also be used to explore the ubiquitin profile of identified interactors. Furthermore, we hope to quantify levels of translation with and without Nedd4-1 activity inhibitors to learn more about Nedd4-1's possible role in synthesis in the neuron.

Of our stimulated conditions, we find that NMDA receptor stimulation causes Nedd4-1 to associate with synaptic pathways. This was surprising given early findings in our lab that suggested Nedd4-1 recruitment may be an NMDA-independent phenotype (Scudder et al. 2014). Even so, these new findings do align with what is described in the field. There is a large breadth of literature describing E3 ligases' roles in synaptic function and health. E3 ligases are known to play a role in synaptic plasticity (Pick JE. et al. 2012; Lee KY. et al. 2018), AMPAR trafficking (Schwarz LA. et al. 2010; Kumari P. et al. 2017; Srinivasan B. et al. 2021; Olabarria M. et al. 2019; Colledge M. et al. 2003), spine density and morphology (Dindot SV. et al. 2008; Hung AY. et al. 2010; Brace EJ. et al. 2014; Tsai NP. et al. 2017; Tsai NP. et al. 2012; Mir S. et al. 2014), and neuromuscular junction formation (Li L. et al. 2016; Xing G. et al. 2019; Chen A. et al. 2020). Previous labs have also shown NMDA receptors recruiting E3 ligases to the synapse to affect synaptic function (Sharma G. et al. 2022; Ma Q. et al. 2017; Bianchetta MJ. et al. 2011). Our data showing NMDA driving Nedd4-1 to associate with synaptic organization and anchoring proteins fits well with the

literature as well as our immunofluorescent experiments showing NMDA-dependent Nedd4-1 colocalization with PSD-95. The drawback to our experiments remains that none of these techniques validate a physical interaction between Nedd4-1 and its interactors. We plan to answer this question using co-immunoprecipitation. Next, we explore a possible functional role of NMDA induced recruitment of Nedd4-1 to the synapse. Using biochemical assays, we first show that NMDA drives the reduction of surface GluA1 but not GluA2. This downregulation of surface receptors is then blocked by pharmacological inhibition of Nedd4-1 activity using the drug 1B-I3C. This indicates that Nedd4-1 may be crucial to NMDA dependent downregulation of surface GluA1 but not GluA2 containing AMPARs. Together this data suggests NMDARs are recruiting and sequestering Nedd4-1 at the synapse to possibly interact with structural proteins there as well as regulate GluA2 lacking Ca^{2+} permeable AMPARs at the surface.

F. Acknowledgments

Chapter 4's work was supported by the The Sanford Burnham Prebys Proteomics Core. Thank you to Dr. Gene Yeo for providing the NES-APEX2-GFP used to clone our APEX2-HA-Nedd4-1 constructs. Thank you to Dr. Kim Dore's lab for contributing electrophysiological recordings using Nedd4-1 inhibitor, 1B-I3C.

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

G. Figures and Tables

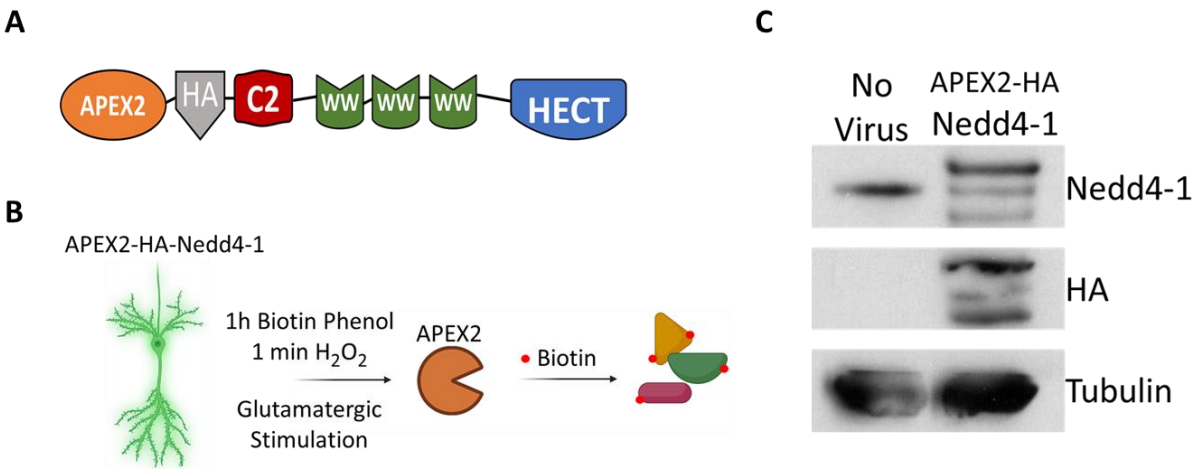

Figure 1 APEX2-HA-Nedd4-1 expresses in dissociated cortical neurons.
A) Model of rat Nedd4-1 N-terminally tagged with APEX2 and HA epitope-tag cloned in attenuated Sindbis virus. B) Schematic of APEX2-mediated proximity labeling in primary rat dissociated cortical neurons. C) Biochemical validation of APEX2-HA-Nedd4-1 expression in neurons. Representative western blots of lysates from dissociated cortical neuronal cultures (DIV 18) virally transduced with Sindbis (attenuated) APEX2-HA-Nedd4-1 (expression from attenuated virus limited to 16-20h). Blots were probed for Nedd4-1, HA and Tubulin.

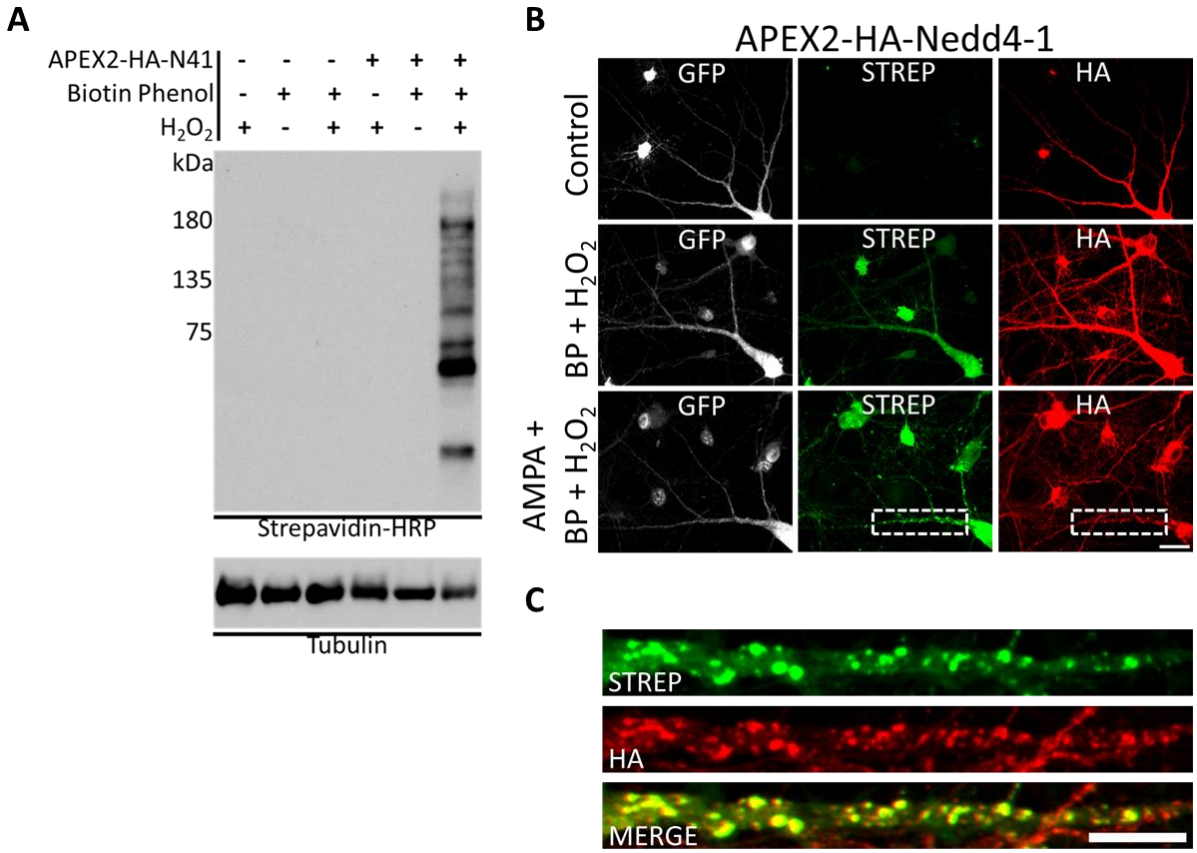

Figure 2 Validation of APEX2 proximity labeling enzymatic function and spatial resolution.

A) Representative western blot of lysates from dissociated cortical neuronal cultures (DIV 18) virally transduced with Sindbis (attenuated) APEX2-HA-Nedd4-1 (expression lasted 16-20h). APEX2-HA-Nedd4-1 biotinylated proteins are only observed with Biotin phenol and H_2O_2 treatment. Uninfected and infected cultures were treated with biotin phenol (500 µM 1 r), H_2O_2 (1 µM for 60 sec), or both. Cell lysates were run on SDS-PAGE gels and Blots were then probed with Streptavidin-HRP. B) Representative immunofluorescent images of dissociated hippocampal neurons (DIV 18–21) expressing APEX2-HA-Nedd4-1 and treated with biotin phenol (BP) + H_2O_2, BP + H_2O_2 and AMPA (10 µM for 10 min) or left untreated. Representative maximum z-projected confocal images. As depicted, Strep-568 signal (middle panels) is only found in APEX2-HA-Nedd4-1 expressing cells treated with biotin phenol (500uM 1hr) and H_2O_2. Proximity labeled and AMPA treated cells show that the APEX2 enzyme does not disrupt Nedd4-1's ability to redistribute in response to stimulation C) Representative straightened dendrite segments from proximity labeled and AMPA treated cells show the tight spatial resolution of APEX2 biotin labeling.

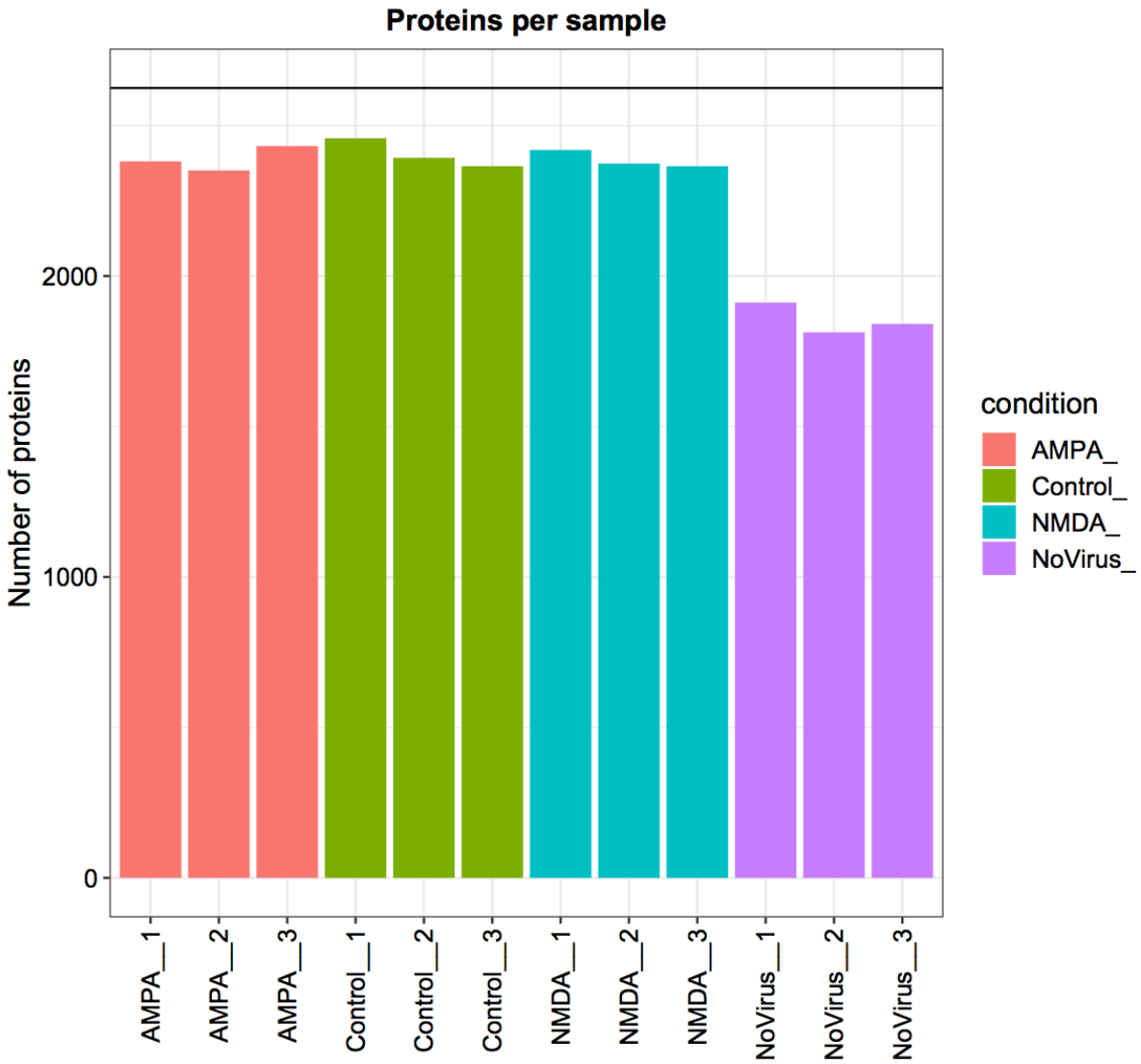

Figure 3 Validation of biotin purification and mass spectrometry analysis.
Bar plot representing the number of proteins identified per sample. The conditions include cells treated with either AMPA, NMDA, Control, and No Virus. All three replicates from each condition is represented. Each condition is colored according to legend.

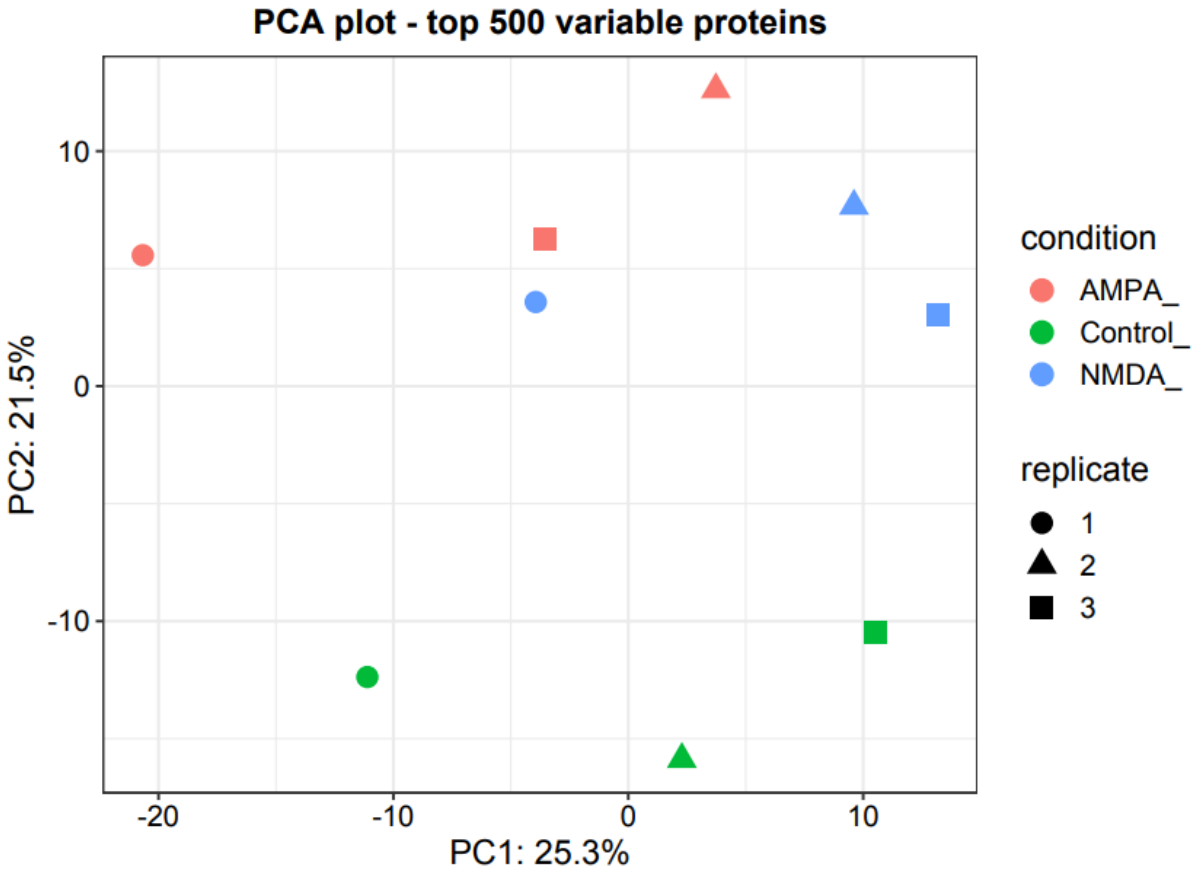

Figure 4 Principal component analysis of activity dependent changes in the Nedd4-1 interactome

Principal Component analysis (PCA) of the top 500 differentially expressed proteins across the AMPA, NMDA, and control treated conditions. Each point represents one biological replicate. The color indicates the condition. The first two principal components, PC1 and PC2, are graphed on the x and y axes, respectively. The three conditions show noticeable separation, indicating that their distinct protein expression profiles across the conditions.

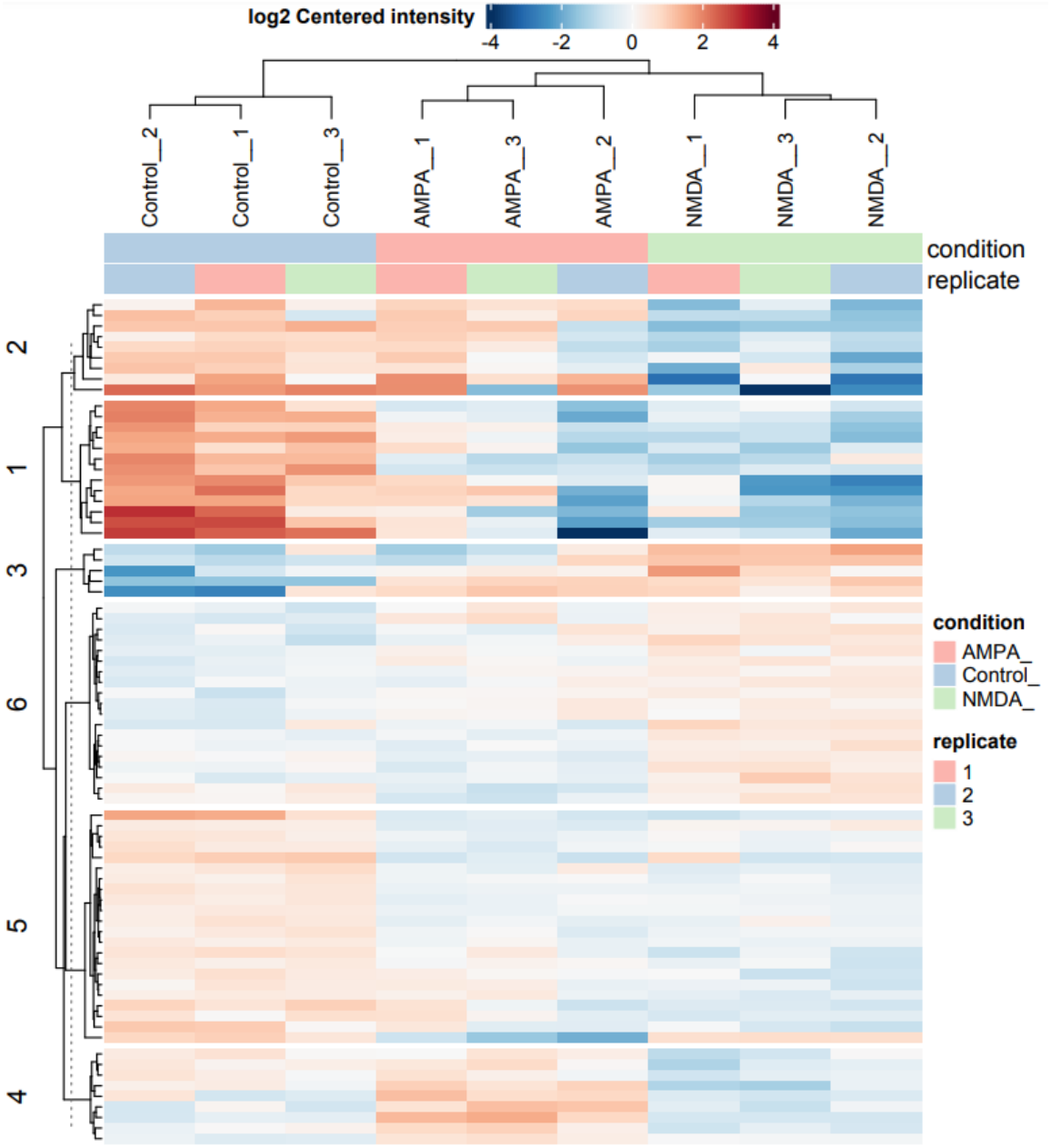

Figure 5 Heatmap of hierarchical clustering of proximity labeling mass spectrometry hits

Heatmap representation of all differentially expressed proteins in all samples from the APEX2-HA-Nedd4-1 BioID dataset. Hierarchical clustering was performed on the 2526 proteins identified in the proximity-dependent labeling and the results are displayed as heat-map clustering of the intensities of proteins with significant change of abundance. Colorbar indicates the log2 centered intensity (protein abundance). All three conditions (Control, AMPA and NMDA) with three biological replicates each were used for k-means cluster analysis. Clustered samples indicate samples with similar protein profiles, whereas clustered proteins indicate proteins with similar abundances.

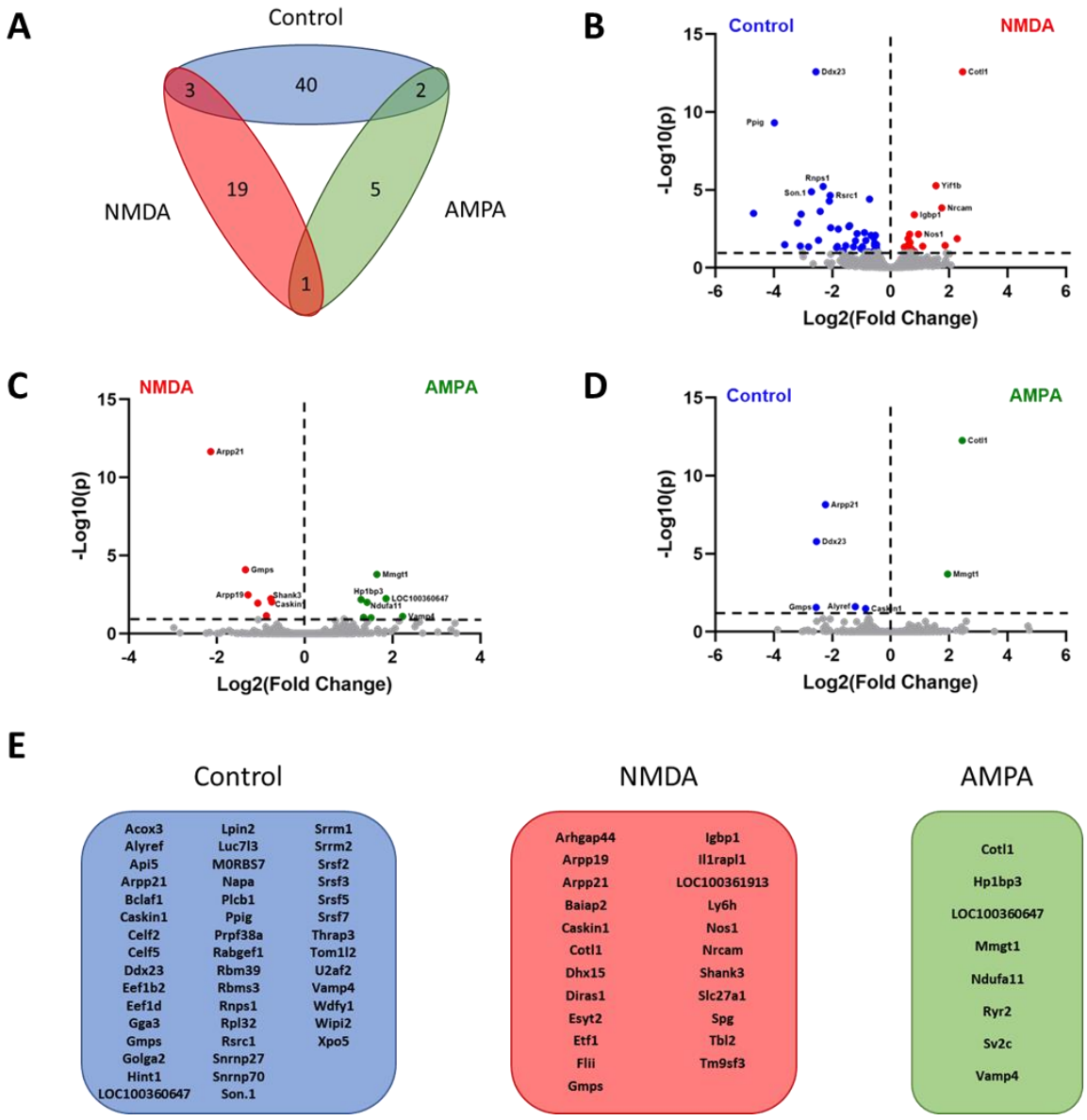

Figure 6 Mass spectrometry analysis identifies activity dependent changes in the Nedd4-1 interactome

A) Vin diagram of the number of enriched hits (EHs) in each condition and their overlap. B-D) Volcano plots show mass spec hits enriched in Control, AMPA, or NMDA with a false discovery rate (FDR) <0.1 with colored circles above a dotted FDR line [B, C, D]. The top 5 significant values are labeled with their gene ID. E) List of EHs with an FDR <0.1 in each condition.

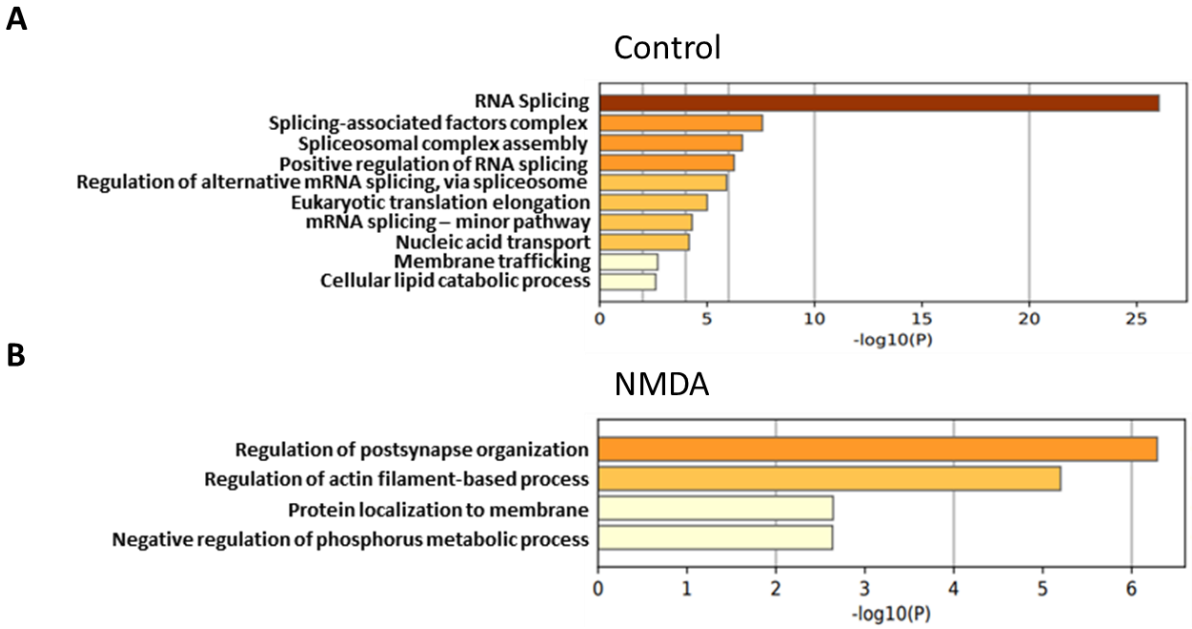

Figure 7 Pathway enrichment of Nedd4-1 interactomes.
Bar graph of enriched terms (colored by p-values) in both A) control and B) NMDA conditions.

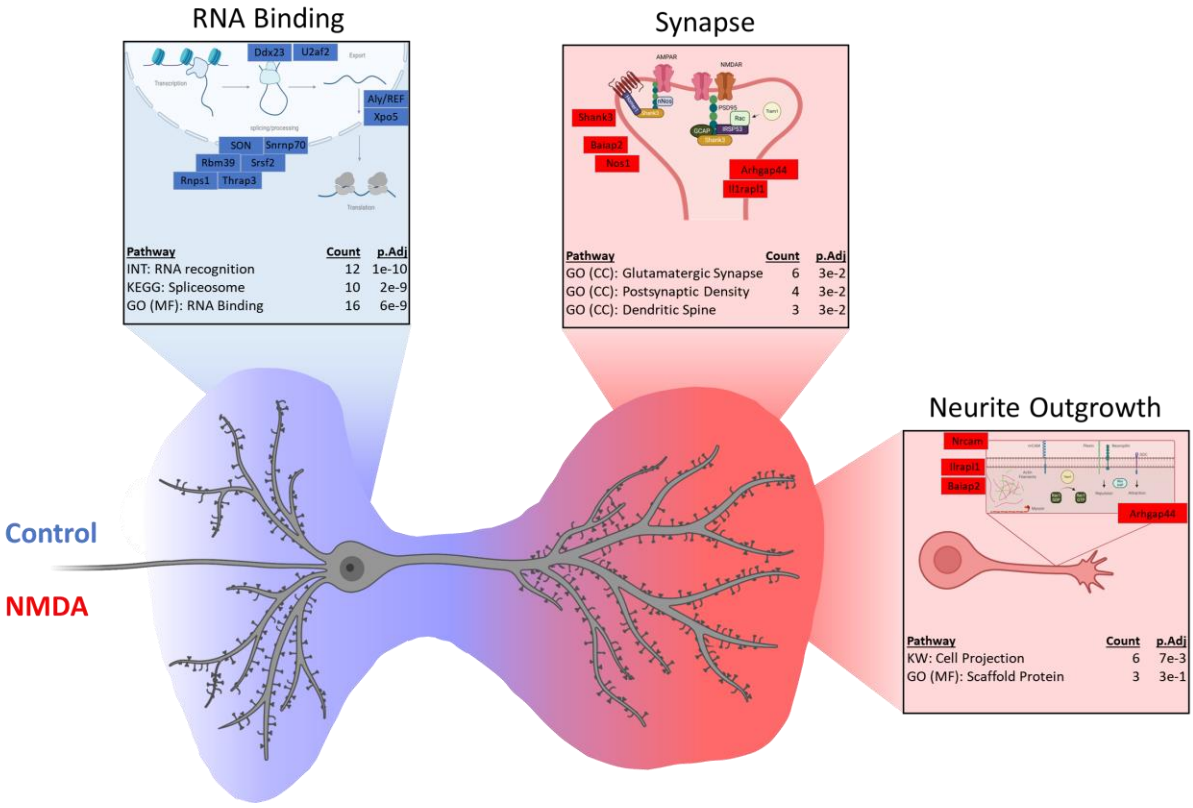

Figure 8 Model of Nedd4-1 interaction in the neuron.
Model depicts graphical representation of pathways enriched in control and NMDA stimulated conditions. Images contain relevant gene names represented in each pathway. Enriched hits from Control and NMDA were analyzed using the DAVID bioinformatics site. DAVID calculates enriched pathways based on the input list of genes and clusters to similar pathways. Depicted in each box is the top biological and molecular pathways per cluster with the gene counts and adjusted p-values. Images generated with BioRender.

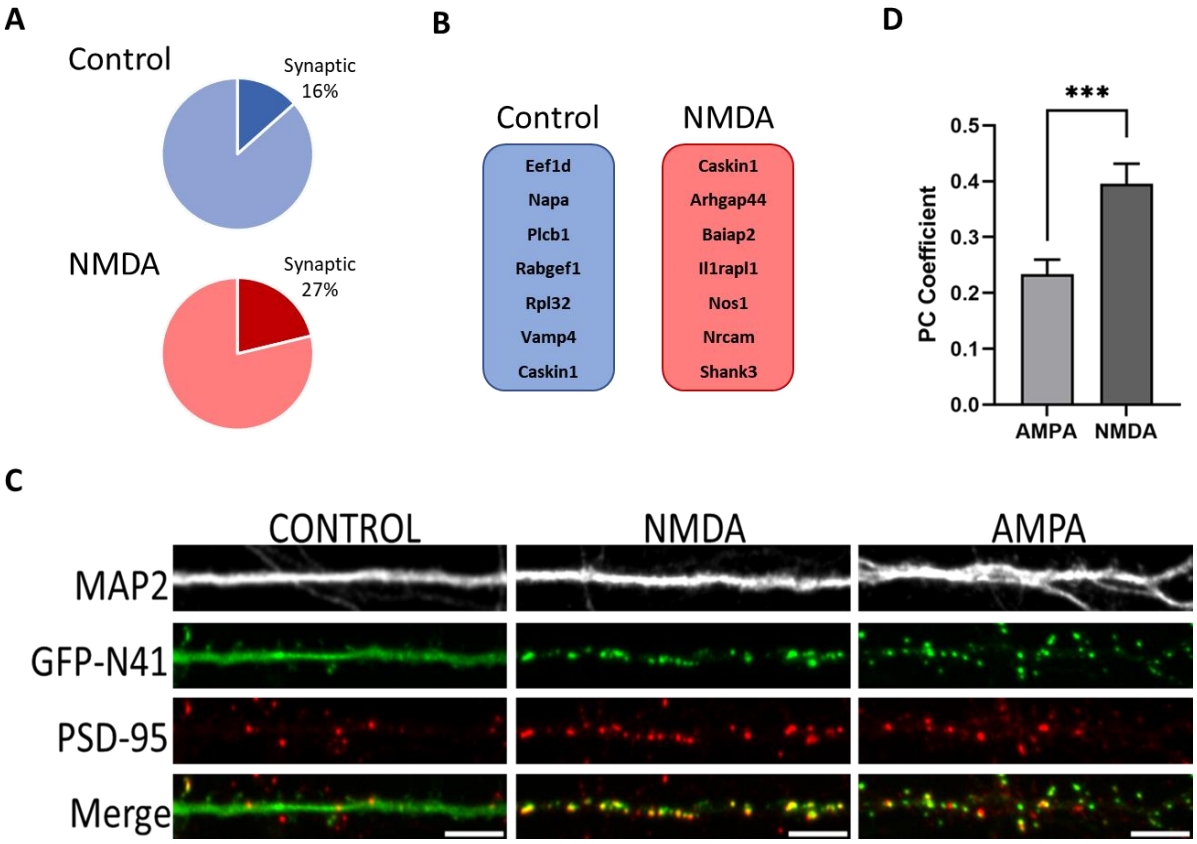

Figure 9 NMDA causes Nedd4-1 to associate with the synapse.

A) Pie chart quantifying the percentage of known synaptic hits out of the total enriched protein hits for control and NMDA treated conditions and B) list of synaptic hits in both control and NMDA treated conditions. C) Representative immunofluorescent images of dissociated hippocampal neurons 18–21 DIV expressing GFP-tagged Nedd4-1 (sindbis) for 16-20h and treated with AMPA (10 μM) 10 min (n=27) or NMDA (50 μM) for 10 min (n=17). GFP-Nedd4-1 is shown in green, PSD-95 in red, and MAP2 in grey. Representative maximum z-projected confocal images of 50 μm segments of dendrite are depicted. D) the JACoP ImageJ plugin was used to quantify the pearson's correlation coefficient between the green and red channels. Statistical analysis was performed using t-test statistical analysis.

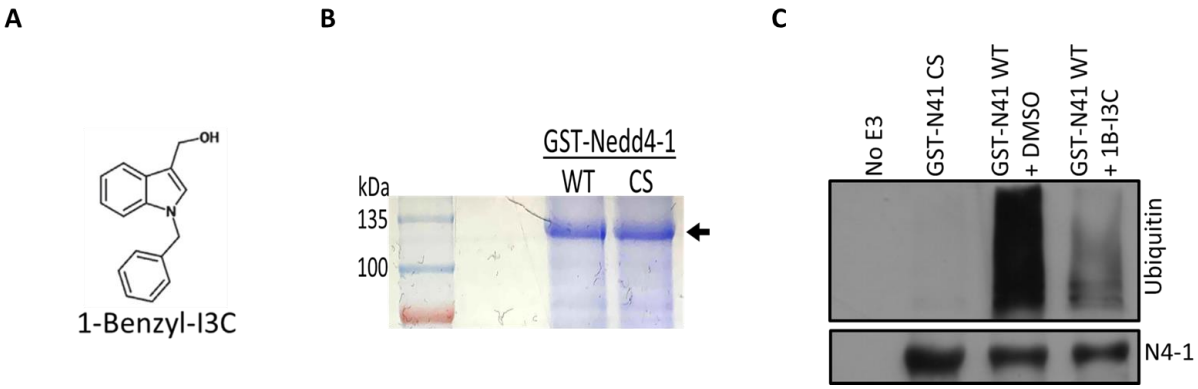

Figure 10 Validation of the pharmacological inhibition of Nedd4-1 with 1B-I3C.
A) Structure of the Nedd4-1 inhibitor 1-benzyl-indole-3-carbinol (1-Benzyl-I3C). B) Coomassie stain of bacterially expressed rat GST-Nedd4-1 wild type (WT) and catalytically inactive (CS) mutant. C) 1-Benzyl-I3C inhibits Nedd4-1 in vitro. Reaction mixtures containing 100 nM 6xHis-E1, 0.5 μM 6xHis-Ubc7, 5 μM 6xHis-Ub, 2 mM ATP, and either No E3, rat GST-Nedd4-1 WT or CS were incubated at room temp for 2hr and analyzed by immunoblotting with anti-Ub.

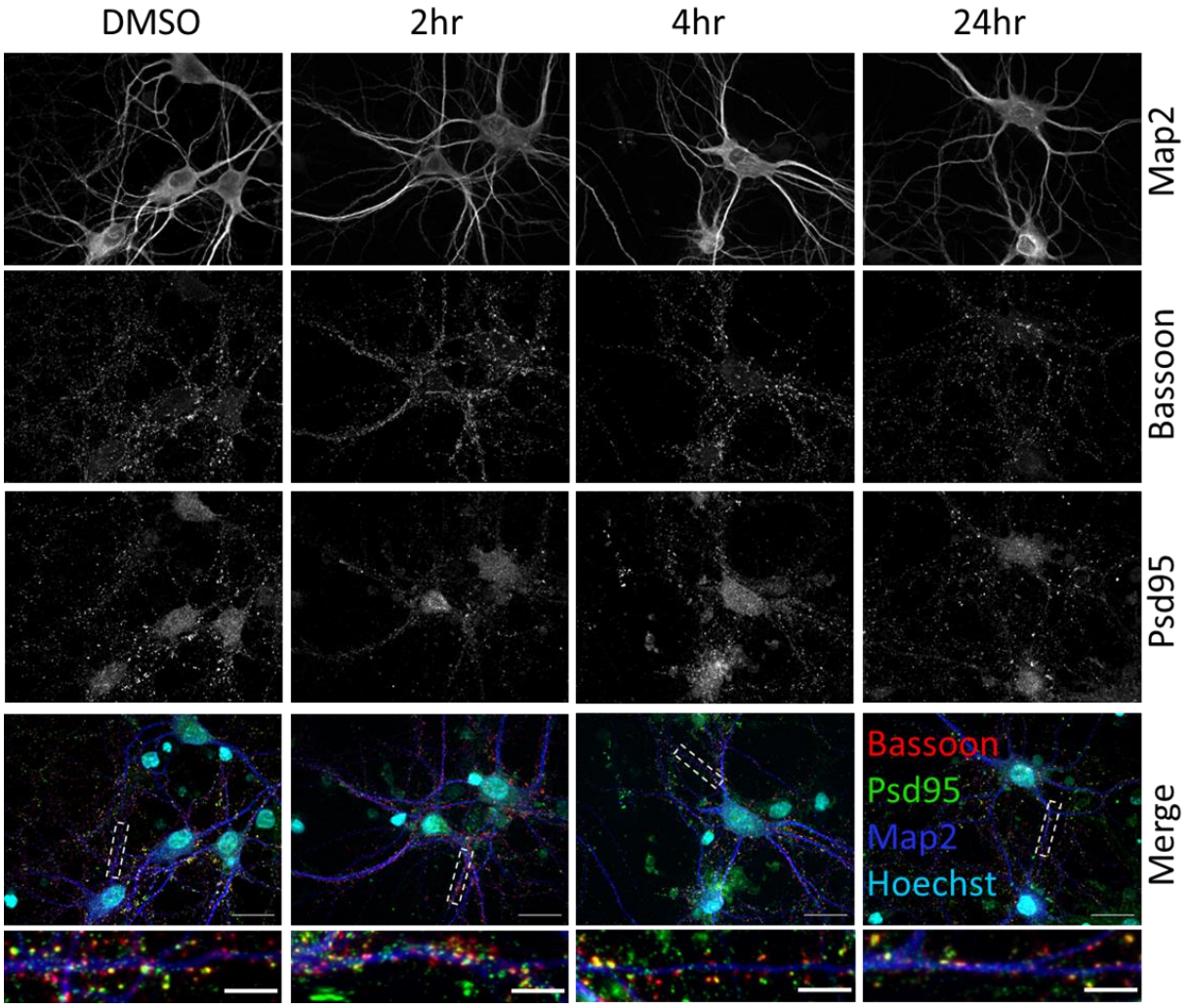

Figure 11 1B-I3C does not affect the health of neuronal cultures.
Representative immunofluorescent images dissociated hippocampal neurons (18–21 DIV) co-stained with Bassoon, PSD-95 and MAP2 antibodies. Representative maximum z-projected confocal images of whole cell and straightened dendrites are depicted. Scale bar is 20 μm and 10 μm, respectively.

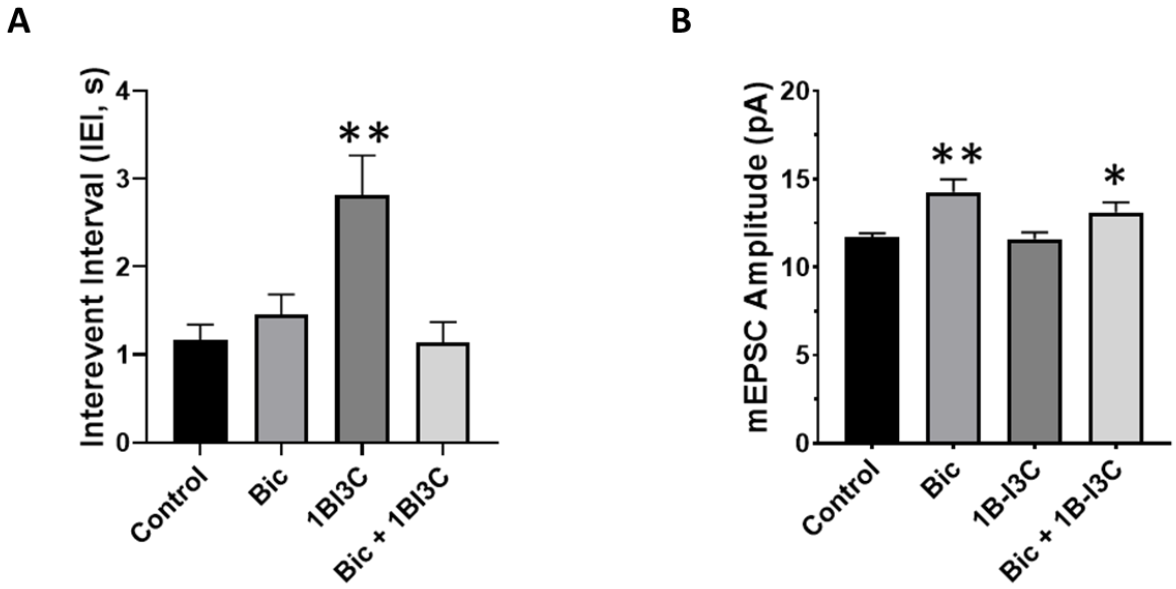

Figure 12 Inhibition of Nedd4-1 by 1-Benzyl I3C decreases frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs).

A) Average Inter-event interval (IEI) of spontaneous miniature EPCSs (mEPSC) recorded in 18-21DIV hippocampal neurons. B) Average amplitude of spontaneous miniature EPCSs (mEPSC) recorded in 18-21DIV hippocampal neurons. Primary hippocampal neurons were pre- treated overnight (16-18h) with either vehicle (DMSO), 20 μM bicuculline (Tocris), 40 μM 1-Benzyl-I3C (ChemDiv) or 20 μM bicuculline plus 40 μM 1-Benzyl-I3C. n = 22, 22, 26 and 22 cells recorded, respectively. Graphs depict mean ± SEM. *p<0.05, **p<0.01, Student's t test. Comparison to control treated group.

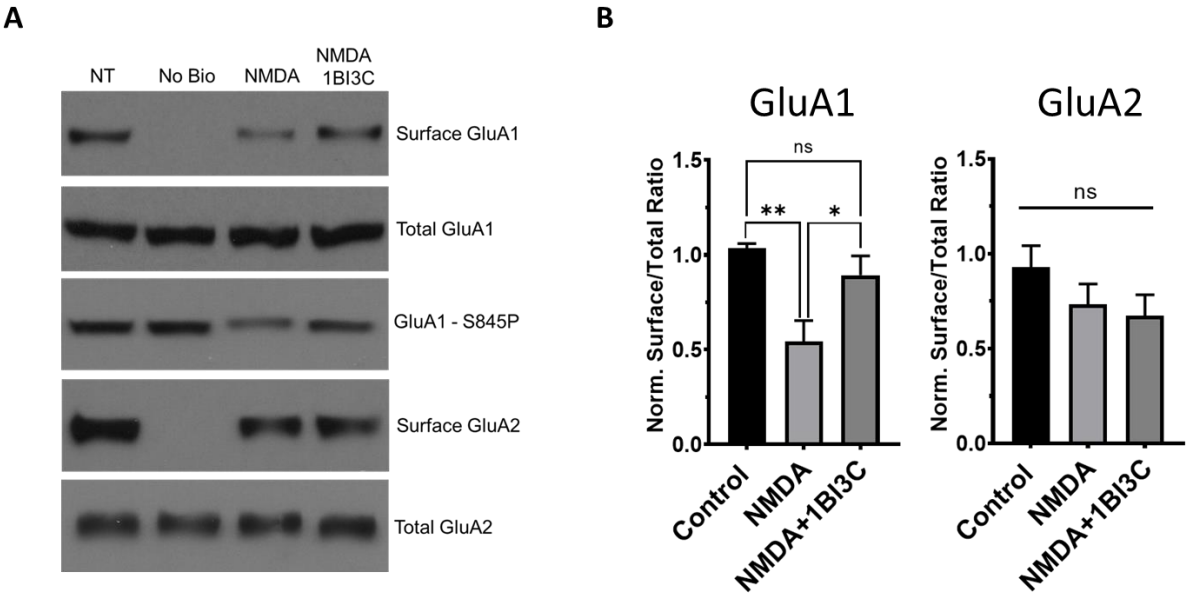

Figure 13 Nedd4-1 activity is necessary for NMDA induced reduction in surface GluA1. Dissociated cortical neuronal cultures (DIV 18-21) were pre-treated with or without 1-Benzyl-I3C for 2-4hr and then NMDA was added for 3 min, subsequently washed out and returned to conditioned media containing 1-Benzyl-I3C or not for 45 min recovery period and then surface biotinylated with NHS-LC-Biotin. Cells not treated with NMDA with and without biotinylation served as control (See Biotinylation Assays in Experimental Proceedures). Biotinylated proteins from resulting lysates were captured by neutravidin-agarose. A) Surface proteins and total lysates were probed in quantitative western blot analysis with GluA1, GluA1-S845, and GluA2 antibodies. B) Bar graphs from quantification of immunoblots depict surface/total ratio for GluA1 and GluA2, respectively. Statistical significance determined by One Way ANOVA. $p < 0.05$ and 0.01 are represented by * and **, respectively. n = 4 biological replicates.

Figure 14 Summary of enriched hits with possible Nedd4-1 interacting site and neurological disease association.

Table presents the protein name, gene ID, and uniprot accession ID, what condition a hit is enriched in, If a protein hit has a PPxY WW domain binding motif, and if the hit has a disease association. Only enriched hits were analyzed. PPxY motifs were identified through manual analysis of the amino acid sequence of each hit. Disease association was described through an extensive literature of each protein hit and possible association with 9 different neurological disorders: Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), Multiple Sclerosis (MS), Glioma, Autism Spectrum Disorder (ASD), Schizophrenia (SZ), or Major Depressive Disorder (MDD). Bolded terms represent protein hits with more than 5 primary research articles linking that protein hit to the bolded disease state.

	Gene Name	ID	Protein Name	Enriched In	PPxY Motif	Disease Association
1	Acox3	F1M9A7	Acyl-coenzyme A oxidase	Control	N	HD
2	Alyref	D3ZXH7	Aly/REF export factor	Control	N	**ALS,** AD, MS
3	Api5	B1WC49	Api5 protein (Apoptosis inhibitor 5)	Control	Y	AD, **glioma**
4	Arhgap44	F1LQX4	Rho GTPase-activating protein 44 (Rho-type GTPase-activating protein RICH2)	NMDA	Y	
5	Arpp19	Q712U5	cAMP-regulated phosphoprotein 19 (ARPP-19)	NMDA	N	PD, AD, glioma
6	Arpp21	A0A0G2JVX9	cAMP regulated phosphoprotein 21	Control, NMDA	Y	ALS, AD, PD, glioma
7	Baiap2	Q6GMN2	Brain-specific angiogenesis inhibitor 1-associated protein 2 (BAI-associated protein 2)	NMDA	Y	AD, HD
8	Bclaf1	B1WC16	BCL2-associated transcription factor 1 (Bclaf1 protein)	Control	N	MS
9	Celf2	Q792H5	CUGBP Elav-like family member 2 (CELF-2)	Control	N	glioma, AD
10	Cotl1	B0BNA5	Coactosin-like protein	AMPA, NMDA	N	AD
11	Diras1	D4A304	DIRAS family GTPase 1	NMDA	N	PD
12	Eef1b2	B5DEN5	Elongation factor 1-beta	Control	N	AD
13	Eef1d	Q68FR9	Elongation factor 1-delta (EF-1-delta)	Control	N	glioma, PD, ALS
14	Esyt2	D3ZJ32	Extended synaptotagmin 2	NMDA	Y	AD
15	Etf1	Q5U2Q7	Eukaryotic peptide chain release factor subunit 1 (Eukaryotic release factor 1) (eRF1)	NMDA	N	glioma
16	Flii	Q5RKI5	Flightless I homolog (Drosophila)	NMDA	N	glioma, PD
17	Gga3	D3ZHG8	Golgi associated, gamma adaptin ear containing, ARF binding protein 3	Control	N	**AD**
18	Gmps	A0A0G2K2C2	GMP synthase (glutamine-hydrolyzing) (EC 6.3.5.2)	Control, NMDA	N	AD
19	Hint1	P62959	Adenosine 5'-monophosphoramidase HINT1 (EC 3.9.1.-) (Histidine triad nucleotide-binding protein 1) (Protein kinase C inhibitor 1) (Protein kinase C-interacting protein 1) (PKCI-1)	Control	N	AD
20	Il1rapl1	A0A096MJW6	Interleukin-1 receptor accessory protein-like 1 (X-linked interleukin-1 receptor accessory protein-like 1)	NMDA	N	**ASD**
21	Luc7l3	D3ZFB2	LUC7-like 3 pre-mRNA splicing factor	Control	N	AD
22	Ly6h	A0A096MJ90	Lymphocyte Antigen 6 Family Member H	NMDA	N	AD
23	Nos1	D3ZEW7	Nitric oxide synthase, brain (EC 1.14.13.39)	NMDA	N	**AD, PD,** HD
24	Nrcam	A0A0G2K3Q5	Neuronal cell adhesion molecule	NMDA	N	**AD,** glioma
25	Plcb1	P10687	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 (EC 3.1.4.11)	Control	N	AD, MS
26	Rabgef1	G3V631	RAB guanine nucleotide exchange factor (GEF) 1 (Predicted)	Control	N	glioma, PD
27	Rpl32	P62912	60S ribosomal protein L32	Control	N	PD
28	Rsrc1	Q5PPJ2	Serine/Arginine-related protein 53 (SRrp53) (Arginine/serine-rich coiled-coil protein 1)	Control	N	**SZ,** MDD, glioma
29	Ryr2	B0LPN4	Ryanodine receptor 2 (RYR-2)	AMPA	Y	**AD, PD, ASD, SZ**
30	Shank3	Q9JLU4	SH3 and multiple ankyrin repeat domains protein 3 (Shank3)	NMDA	N	**ASD, AD**
31	Slc27a1	Q6GMM8	Solute carrier family 27 (Fatty acid transporter), member 1	NMDA	N	AD
32	Snrnp70	B2RZ74	U1 small nuclear ribonucleoprotein 70 kDa	Control	N	ALS, AD
33	Son.1	F1MAQ7	SON DNA and RNA binding protein	Control	Y	
34	Srrm2	A0A0G2K2M9	Serine/arginine repetitive matrix 2	Control	N	**PD,** AD
35	Srsf2	Q6PDU1	Serine/arginine-rich splicing factor 2 (Splicing component, 35 kDa)	Control	N	AD
36	Srsf3	A0A0U1RRV7	Serine and arginine rich splicing factor 3	Control	N	glioma
37	Srsf5	Q09167	Serine/arginine-rich splicing factor 5	Control	N	AD
38	Sv2c	Q9Z2I6	Synaptic vesicle glycoprotein 2C (Synaptic vesicle protein 2C)	AMPA	N	**PD**, HD
39	Tom1l2	A0A0G2K9L2	Target of myb1 like 2 membrane trafficking protein	Control	N	AD
40	U2af2	F2Z3T9	Splicing factor U2AF subunit (U2 snRNP auxiliary factor large subunit)	Control	N	glioma
41	Vamp4	A0A096MJ99	Vesicle-associated membrane protein 4	Control, AMPA	N	**PD,** AD
42	Wdfy1	A0A0G2K3L8	WD repeat and FYVE domain containing 1	Control	N	AD
43	Xpo5	D3ZQE8	Exportin 5	Control	N	AD, ALS

H. References

Swatek KN, Komander D. Ubiquitin modifications. Cell Res. 2016;26(4):399-422. doi:10.1038/cr.2016.39

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP. A proteomics approach to understanding protein ubiquitination. Nat Biotechnol. 2003;21(8):921-926. doi:10.1038/nbt849

Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP. Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell. 2011;44(2):325-340. doi:10.1016/j.molcel.2011.08.025

Wagner SA, Beli P, Weinert BT, Nielsen ML, Cox J, Mann M, Choudhary C. A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. Mol Cell Proteomics. 2011;10(10):M111.013284. doi:10.1074/mcp.M111.013284

Cunningham CN, Baughman JM, Phu L, Tea JS, Yu C, Coons M, Kirkpatrick DS, Bingol B, Corn JE. USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria. Nat Cell Biol. 2015;17(2):160-169. doi:10.1038/ncb3097

Wickliffe KE, Williamson A, Meyer HJ, Kelly A, Rape M. K11-linked ubiquitin chains as novel regulators of cell division. Trends Cell Biol. 2011;21(11):656-663. doi:10.1016/j.tcb.2011.08.008

Gatti M, Pinato S, Maiolica A, Rocchio F, Prato MG, Aebersold R, Penengo L. RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage. Cell Rep. 2015;10(2):226-238. doi:10.1016/j.celrep.2014.12.021

Fiil BK, Damgaard RB, Wagner SA, Keusekotten K, Fritsch M, Bekker-Jensen S, Mailand N, Choudhary C, Komander D, Gyrd-Hansen M. OTULIN restricts Met1-linked ubiquitination to control innate immune signaling. Mol Cell. 2013;50(6):818-830. doi:10.1016/j.molcel.2013.06.004

Licchesi JD, Mieszczanek J, Mevissen TE, Rutherford TJ, Akutsu M, Virdee S, El Oualid F, Chin JW, Ovaa H, Bienz M, Komander D. An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains. Nat Struct Mol Biol. 2011;19(1):62-71. Published 2011 Dec 11. doi:10.1038/nsmb.2169

Jin J, Xie X, Xiao Y, Hu H, Zou Q, Cheng X, Sun SC. Epigenetic regulation of the expression of Il12 and Il23 and autoimmune inflammation by the deubiquitinase Trabid. Nat Immunol. 2016;17(3):259-268. doi:10.1038/ni.3347

Yuan WC, Lee YR, Lin SY, Chang LY, Tan YP, Hung CC, Kuo JC, Liu CH, Lin MY, Xu M, Chen ZJ, Chen RH. K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking. Mol Cell. 2014;54(4):586-600. doi:10.1016/j.molcel.2014.03.035

Yalcin E, Beker MC, Turkseven S, Caglayan B, Gurel B, Kilic U, Caglayan AB, Kalkan R, Baykal AT, Kelestemur T, Kilic E. Evidence that melatonin downregulates Nedd4-1 E3 ligase and its role in cellular survival. Toxicol Appl Pharmacol. 2019;379:114686. doi:10.1016/j.taap.2019.114686

Ding X, Jo J, Wang CY, Cristobal CD, Zuo Z, Ye Q, Wirianto M, Lindeke-Myers A, Choi JM, Mohila CA, Kawabe H, Jung SY, Bellen HJ, Yoo SH, Lee HK. The Daam2-VHL-Nedd4 axis governs developmental and regenerative oligodendrocyte differentiation. *Genes Dev*. 2020;34(17-18):1177-1189. doi:10.1101/gad.338046.120

Zhu J, Lee KY, Jong TT, Tsai NP. C2-lacking isoform of Nedd4-2 regulates excitatory synaptic strength through GluA1 ubiquitination-independent mechanisms. J Neurochem. 2019;151(3):289-300. doi:10.1111/jnc.14840

Dong Z, Chen W, Chen C, Wang H, Cui W, Tan Z, Robinson H, Gao N, Luo B, Zhang L, Zhao K, Xiong WC, Mei L. CUL3 Deficiency Causes Social Deficits and Anxiety-like Behaviors by Impairing Excitation-Inhibition Balance through the Promotion of Cap-Dependent Translation. Neuron. 2020;105(3):475-490.e6. doi:10.1016/j.neuron.2019.10.035

Howley BV, Mohanty B, Dalton A, Grelet S, Karam J, Dincman T, Howe PH. The ubiquitin E3 ligase ARIH1 regulates hnRNP E1 protein stability, EMT and breast cancer progression. Oncogene. 2022;41(12):1679-1690. doi:10.1038/s41388-022-02199-9

Zhai Y, Huang X, Zhang K, Huang Y, Jiang Y, Cui J, Zhang Z, Chiu CKC, Zhong W, Li G. Spatiotemporal-resolved protein networks profiling with photoactivation dependent proximity labeling. Nat Commun. 2022;13(1):4906. Published 2022 Aug 20. doi:10.1038/s41467-022-32689-z

Cao X, Shami Shah A, Sanford EJ, Smolka MB, Baskin JM. Proximity Labeling Reveals Spatial Regulation of the Anaphase-Promoting Complex/Cyclosome by a Microtubule Adaptor. ACS Chem Biol. 2022;17(9):2605-2618. doi:10.1021/acschembio.2c00527

Zuzow N, Ghosh A, Leonard M, Liao J, Yang B, Bennett EJ. Mapping the mammalian ribosome quality control complex interactome using proximity labeling approaches. Mol Biol Cell. 2018;29(10):1258-1269. doi:10.1091/mbc.E17-12-0714

Coyaud E, Mis M, Laurent EM, Dunham WH, Couzens AL, Robitaille M, Gingras AC, Angers S, Raught B. BioID-based Identification of Skp Cullin F-box (SCF)β-TrCP1/2 E3 Ligase Substrates. Mol Cell Proteomics. 2015;14(7):1781-1795. doi:10.1074/mcp.M114.045658

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Lin A, Hou Q, Jarzylo L, Amato S, Gilbert J, Shang F, Man HY. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. J Neurochem. 2011;119(1):27-39. doi:10.1111/j.1471-4159.2011.07221.x

Hou Q, Gilbert J, Man HY. Homeostatic regulation of AMPA receptor trafficking and degradation by light-controlled single-synaptic activation. Neuron. 2011;72(5):806-818. doi:10.1016/j.neuron.2011.10.011

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Wei J, Xiong Z, Lee JB, Cheng J, Duffney LJ, Matas E, Yan Z. Histone Modification of Nedd4 Ubiquitin Ligase Controls the Loss of AMPA Receptors and Cognitive Impairment Induced by Repeated Stress. J Neurosci. 2016;36(7):2119-2130. doi:10.1523/JNEUROSCI.3056-15.2016

Gautam V, Trinidad JC, Rimerman RA, Costa BM, Burlingame AL, Monaghan DT. Nedd4 is a specific E3 ubiquitin ligase for the NMDA receptor subunit GluN2D. Neuropharmacology. 2013;74:96-107. doi:10.1016/j.neuropharm.2013.04.035

Lee S, Park S, Lee H, Han S, Song JM, Han D, Suh YH. Nedd4 E3 ligase and beta-arrestins regulate ubiquitination, trafficking, and stability of the mGlu7 receptor. Elife. 2019;8:e44502. Published 2019 Aug 2. doi:10.7554/eLife.44502

Persaud A, Alberts P, Hayes M, Guettler S, Clarke I, Sicheri F, Dirks P, Ciruna B, Rotin D. Nedd4-1 binds and ubiquitylates activated FGFR1 to control its endocytosis and function. EMBO J. 2011;30(16):3259-3273. Published 2011 Jul 15. doi:10.1038/emboj.2011.234

Kawabe H, Neeb A, Dimova K, Young SM Jr, Takeda M, Katsurabayashi S, Mitkovski M, Malakhova OA, Zhang DE, Umikawa M, Kariya K, Goebbels S, Nave KA, Rosenmund C, Jahn O, Rhee J, Brose N. Regulation of Rap2A by the ubiquitin ligase Nedd4-1 controls neurite development. Neuron. 2010;65(3):358-372. doi:10.1016/j.neuron.2010.01.007

Fu Z, Lee SH, Simonetta A, Hansen J, Sheng M, Pak DT. Differential roles of Rap1 and Rap2 small GTPases in neurite retraction and synapse elimination in hippocampal spiny neurons. J Neurochem. 2007;100(1):118-131. doi:10.1111/j.1471-4159.2006.04195.x

Taira K, Umikawa M, Takei K, Myagmar BE, Shinzato M, Machida N, Uezato H, Nonaka S, Kariya K. The Traf2- and Nck-interacting kinase as a putative effector of Rap2 to regulate actin cytoskeleton. J Biol Chem. 2004;279(47):49488-49496. doi:10.1074/jbc.M406370200

Christie KJ, Martinez JA, Zochodne DW. Disruption of E3 ligase NEDD4 in peripheral neurons interrupts axon outgrowth: Linkage to PTEN. Mol Cell Neurosci. 2012;50(2):179-192. doi:10.1016/j.mcn.2012.04.006

Hsia HE, Kumar R, Luca R, Takeda M, Courchet J, Nakashima J, Wu S, Goebbels S, An W, Eickholt BJ, Polleux F, Rotin D, Wu H, Rossner MJ, Bagni C, Rhee JS, Brose N, Kawabe H. Ubiquitin E3 ligase Nedd4-1 acts as a downstream target of PI3K/PTEN-mTORC1 signaling to promote neurite growth. Proc Natl Acad Sci U S A. 2014;111(36):13205-13210. doi:10.1073/pnas.1400737111

Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N. Astroglial networks: a step further in neuroglial and gliovascular interactions. Nat Rev Neurosci. 2010;11(2):87-99. doi:10.1038/nrn2757

Liao CK, Jeng CJ, Wang HS, Wang SH, Wu JC. Lipopolysaccharide induces degradation of connexin43 in rat astrocytes via the ubiquitin-proteasome proteolytic pathway. PLoS One. 2013;8(11):e79350. Published 2013 Nov 13. doi:10.1371/journal.pone.0079350

Liu Y, Oppenheim RW, Sugiura Y, Lin W. Abnormal development of the neuromuscular junction in Nedd4-deficient mice. Dev Biol. 2009;330(1):153-166. doi:10.1016/j.ydbio.2009.03.023

Camera D, Boase NA, Kumar S, Pow DV, Poronnik P. Subtle gait abnormalities in Nedd4 heterozygous mice. Behav Brain Res. 2014;260:15-24. doi:10.1016/j.bbr.2013.11.024

Kwak YD, Wang B, Li JJ, Wang R, Deng Q, Diao S, Chen Y, Xu R, Masliah E, Xu H, Sung JJ, Liao FF. Upregulation of the E3 ligase NEDD4-1 by oxidative stress degrades IGF-1 receptor protein in neurodegeneration. J Neurosci. 2012;32(32):10971-10981. doi:10.1523/JNEUROSCI.1836-12.2012

Rodrigues EM, Scudder SL, Goo MS, Patrick GN. Aβ-Induced Synaptic Alterations Require the E3 Ubiquitin Ligase Nedd4-1. J Neurosci. 2016;36(5):1590-1595. doi:10.1523/JNEUROSCI.2964-15.2016

Guntupalli S, Jang SE, Zhu T, Huganir RL, Widagdo J, Anggono V. GluA1 subunit ubiquitination mediates amyloid-β-induced loss of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem. 2017;292(20):8186-8194. doi:10.1074/jbc.M116.774554

Zhang Y, Guo O, Huo Y, Wang G, Man HY. Amyloid-β Induces AMPA Receptor Ubiquitination and Degradation in Primary Neurons and Human Brains of Alzheimer's Disease. J Alzheimers Dis. 2018;62(4):1789-1801. doi:10.3233/JAD-170879

Tofaris GK, Kim HT, Hourez R, Jung JW, Kim KP, Goldberg AL. Ubiquitin ligase Nedd4 promotes alpha-synuclein degradation by the endosomal-lysosomal pathway. Proc Natl Acad Sci U S A. 2011;108(41):17004-17009. doi:10.1073/pnas.1109356108 https://doi.org/10.1073/pnas.1109356108

Sugeno N, Hasegawa T, Tanaka N, Fukuda M, Wakabayashi K, Oshima R, Konno M, Miura E, Kikuchi A, Baba T, Anan T, Nakao M, Geisler S, Aoki M, Takeda A. Lys-63-linked ubiquitination by E3 ubiquitin ligase Nedd4-1 facilitates endosomal sequestration of internalized α-synuclein. J Biol Chem. 2014;289(26):18137-18151. doi:10.1074/jbc.M113.529461

Mund T, Masuda-Suzukake M, Goedert M, Pelham HR. Ubiquitination of alpha-synuclein filaments by Nedd4 ligases [published correction appears in PLoS One. 2018 Aug 7;13(8):e0202158]. PLoS One. 2018;13(7):e0200763. Published 2018 Jul 18. doi:10.1371/journal.pone.0200763

Won SY, Park JJ, You ST, Hyeun JA, Kim HK, Jin BK, McLean C, Shin EY, Kim EG. p21-activated kinase 4 controls the aggregation of α-synuclein by reducing the monomeric and aggregated forms of α-synuclein: involvement of the E3 ubiquitin ligase NEDD4-1. Cell Death Dis. 2022;13(6):575. Published 2022 Jun 30. doi:10.1038/s41419-022-05030-1

Warnica W, Merico D, Costain G, Alfred SE, Wei J, Marshall CR, Scherer SW, Bassett AS. Copy number variable microRNAs in schizophrenia and their neurodevelopmental gene targets. Biol Psychiatry. 2015;77(2):158-166. doi:10.1016/j.biopsych.2014.05.011

Han C, Cui K, Bi X, Wang L, Sun M, Yang L, Liu L. Association between polymorphism of the NEDD4 gene and cognitive dysfunction of schizophrenia patients in Chinese Han population. BMC Psychiatry. 2019;19(1):405. Published 2019 Dec 18. doi:10.1186/s12888-019-2386-y

Dai B, Pieper RO, Li D, Wei P, Liu M, Woo SY, Aldape KD, Sawaya R, Xie K, Huang S. FoxM1B regulates NEDD4-1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. Cancer Res. 2010;70(7):2951-2961. doi:10.1158/0008-5472.CAN-09-3909

Chuang HY, Hsu LY, Pan CM, Pikatan NW, Yadav VK, Fong IH, Chen CH, Yeh CT, Chiu SC. The E3 Ubiquitin Ligase NEDD4-1 Mediates Temozolomide-Resistant Glioblastoma through PTEN Attenuation and Redox Imbalance in Nrf2-HO-1 Axis. Int J Mol Sci. 2021;22(19):10247. Published 2021 Sep 23. doi:10.3390/ijms221910247

Rimkus TK, Arrigo AB, Zhu D, Carpenter RL, Sirkisoon S, Doheny D, Regua AT, Wong GL, Manore S, Wagner C, Lin HK, Jin G, Ruiz J, Chan M, Debinski W, Lo HW. NEDD4 degrades TUSC2 to promote glioblastoma progression. Cancer Lett. 2022;531:124-135. doi:10.1016/j.canlet.2022.01.029

Zang Y, Dong M, Zhang K, Tian W, Wang Y, Xue F. Bioinformatics analysis of key differentially expressed genes in well and poorly differentiated endometrial carcinoma. Mol Med Rep. 2018;18(1):467-476. doi:10.3892/mmr.2018.8969

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019;10(1):1523. Published 2019 Apr 3. doi:10.1038/s41467-019-09234-6

Hung V, Udeshi ND, Lam SS, Loh KH, Cox KJ, Pedram K, Carr SA, Ting AY. Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. Nat Protoc. 2016;11(3):456-475. doi:10.1038/nprot.2016.018

Mair A, Bergmann DC. Advances in enzyme-mediated proximity labeling and its potential for plant research. Plant Physiol. 2022;188(2):756-768. doi:10.1093/plphys/kiab479

Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat Methods. 2015;12(1):51-54. doi:10.1038/nmeth.3179

Koopmans F, van Nierop P, Andres-Alonso M, Byrnes A, Cijsouw T, Coba MP, Cornelisse LN, Farrell RJ, Goldschmidt HL, Howrigan DP, Hussain NK, Imig C, de Jong APH, Jung H, Kohansalnodehi M, Kramarz B, Lipstein N, Lovering RC, MacGillavry H, Mariano V, Mi H, Ninov M, Osumi-Sutherland D, Pielot R, Smalla KH, Tang H, Tashman K, Toonen RFG, Verpelli C, Reig-Viader R, Watanabe K, van Weering J, Achsel T, Ashrafi G, Asi N, Brown TC, De Camilli P, Feuermann M, Foulger RE, Gaudet P, Joglekar A, Kanellopoulos A, Malenka R, Nicoll RA, Pulido C, de Juan-Sanz J, Sheng M, Südhof TC, Tilgner HU, Bagni C, Bayés À, Biederer T, Brose N, Chua JJE, Dieterich DC, Gundelfinger ED, Hoogenraad C, Huganir RL, Jahn R, Kaeser PS, Kim E, Kreutz MR, McPherson PS, Neale BM, O'Connor V, Posthuma D, Ryan TA, Sala C, Feng G, Hyman SE, Thomas PD, Smit AB, Verhage M. SynGO: An Evidence-Based, Expert-Curated Knowledge Base for the Synapse. Neuron. 2019;103(2):217-234.e4. doi:10.1016/j.neuron.2019.05.002

Quirit JG, Lavrenov SN, Poindexter K, Xu J, Kyauk C, Durkin KA, Aronchik I, Tomasiak T, Solomatin YA, Preobrazhenskaya MN, Firestone GL. Indole-3-carbinol (I3C) analogues are potent small molecule inhibitors of NEDD4-1 ubiquitin ligase activity that disrupt proliferation of human melanoma cells. Biochem Pharmacol. 2017;127:13-27. doi:10.1016/j.bcp.2016.12.007

Yalcin E, Beker MC, Turkseven S, Caglayan B, Gurel B, Kilic U, Caglayan AB, Kalkan R, Baykal AT, Kelestemur T, Kilic E. Evidence that melatonin downregulates Nedd4-1 E3 ligase and its role in cellular survival. Toxicol Appl Pharmacol. 2019;379:114686. doi:10.1016/j.taap.2019.114686

Hatstat AK, Pupi MD, McCafferty DG. Predicting PY motif-mediated protein-protein interactions in the Nedd4 family of ubiquitin ligases. PLoS One. 2021;16(10):e0258315. Published 2021 Oct 12. doi:10.1371/journal.pone.0258315

Persaud A, Alberts P, Amsen EM, Xiong X, Wasmuth J, Saadon Z, Fladd C, Parkinson J, Rotin D. Comparison of substrate specificity of the ubiquitin ligases Nedd4 and Nedd4-2 using proteome arrays. Mol Syst Biol. 2009;5:333. doi:10.1038/msb.2009.85

Manning J, Windley SP, Sandow JJ, Shah SS, Western P, Wilhelm D, Kumar S. Identification of novel interacting partners of the NEDD4 ubiquitin ligase in mouse testis. J Proteomics. 2020;223:103830. doi:10.1016/j.jprot.2020.103830

Lohraseb I, McCarthy P, Secker G, Marchant C, Wu J, Ali N, Kumar S, Daly RJ, Harvey NL, Kawabe H, Kleifeld O, Wiszniak S, Schwarz Q. Global ubiquitinome profiling identifies NEDD4 as a regulator of Profilin 1 and actin remodeling in neural crest cells. Nat Commun. 2022;13(1):2018. Published 2022 Apr 19. doi:10.1038/s41467-022-29660-3

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Plant PJ, Yeger H, Staub O, Howard P, Rotin D. The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca2+-dependent plasma membrane localization. J Biol Chem. 1997;272(51):32329-32336. doi:10.1074/jbc.272.51.32329

Plant PJ, Lafont F, Lecat S, Verkade P, Simons K, Rotin D. Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb. J Cell Biol. 2000;149(7):1473-1484. doi:10.1083/jcb.149.7.1473

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Cano F, Miranda-Saavedra D, Lehner PJ. RNA-binding E3 ubiquitin ligases: novel players in nucleic acid regulation. Biochem Soc Trans. 2010;38(6):1621-1626. doi:10.1042/BST0381621

Choudhury NR, Nowak JS, Zuo J, Rappsilber J, Spoel SH, Michlewski G. Trim25 Is an RNA-Specific Activator of Lin28a/TuT4-Mediated Uridylation. Cell Rep. 2014;9(4):1265-1272. doi:10.1016/j.celrep.2014.10.017

Lokireddy S, Wijesoma IW, Sze SK, McFarlane C, Kambadur R, Sharma M. Identification of atrogin-1-targeted proteins during the myostatin-induced skeletal muscle wasting [published correction appears in Am J Physiol Cell Physiol. 2014 Dec 15;307(12):C1151]. Am J Physiol Cell Physiol. 2012;303(5):C512-C529. doi:10.1152/ajpcell.00402.2011

Swenson SA, Gilbreath TJ, Vahle H, Hynes-Smith RW, Graham JH, Law HC, Amador C, Woods NT, Green MR, Buckley SM. UBR5 HECT domain mutations identified in mantle cell lymphoma control maturation of B cells. Blood. 2020;136(3):299-312. doi:10.1182/blood.2019002102

Sun J, Keim CD, Wang J, Kazadi D, Oliver PM, Rabadan R, Basu U. E3-ubiquitin ligase Nedd4 determines the fate of AID-associated RNA polymerase II in B cells. Genes Dev. 2013;27(16):1821-1833. doi:10.1101/gad.210211.112

Song F, Fan C, Wang X, Goodrich DW. The Thoc1 encoded ribonucleoprotein is a substrate for the NEDD4-1 E3 ubiquitin protein ligase. PLoS One. 2013;8(2):e57995. doi:10.1371/journal.pone.0057995

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014

Pick JE, Malumbres M, Klann E. The E3 ligase APC/C-Cdh1 is required for associative fear memory and long-term potentiation in the amygdala of adult mice. Learn Mem. 2012;20(1):11-20. Published 2012 Dec 14. doi:10.1101/lm.027383.112

Lee KY, Jewett KA, Chung HJ, Tsai NP. Loss of fragile X protein FMRP impairs homeostatic synaptic downscaling through tumor suppressor p53 and ubiquitin E3 ligase Nedd4-2. Hum Mol Genet. 2018;27(16):2805-2816. doi:10.1093/hmg/ddy189

Kumari P, Srinivasan B, Banerjee S. Modulation of hippocampal synapse maturation by activity-regulated E3 ligase via non-canonical pathway. Neuroscience. 2017;364:226-241. doi:10.1016/j.neuroscience.2017.08.057

Srinivasan B, Samaddar S, Mylavarapu SVS, Clement JP, Banerjee S. Homeostatic scaling is driven by a translation-dependent degradation axis that recruits miRISC remodeling. PLoS Biol. 2021;19(11):e3001432. Published 2021 Nov 23. doi:10.1371/journal.pbio.3001432

Olabarria M, Pasini S, Corona C, Robador P, Song C, Patel H, Lefort R. Dysfunction of the ubiquitin ligase E3A Ube3A/E6-AP contributes to synaptic pathology in Alzheimer's disease. Commun Biol. 2019;2:111. Published 2019 Mar 22. doi:10.1038/s42003-019-0350-5

Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron. 2003;40(3):595-607. doi:10.1016/s0896-6273(03)00687-1

Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. Hum Mol Genet. 2008;17(1):111-118. doi:10.1093/hmg/ddm288

Hung AY, Sung CC, Brito IL, Sheng M. Degradation of postsynaptic scaffold GKAP and regulation of dendritic spine morphology by the TRIM3 ubiquitin ligase in rat hippocampal neurons. PLoS One. 2010;5(3):e9842. Published 2010 Mar 24. doi:10.1371/journal.pone.0009842

Brace EJ, Wu C, Valakh V, DiAntonio A. SkpA restrains synaptic terminal growth during development and promotes axonal degeneration following injury. J Neurosci. 2014;34(25):8398-8410. doi:10.1523/JNEUROSCI.4715-13.2014

Tsai NP, Wilkerson JR, Guo W, Huber KM. FMRP-dependent Mdm2 dephosphorylation is required for MEF2-induced synapse elimination. Hum Mol Genet. 2017;26(2):293-304. doi:10.1093/hmg/ddw386

Tsai NP, Wilkerson JR, Guo W, Maksimova MA, DeMartino GN, Cowan CW, Huber KM. Multiple autism-linked genes mediate synapse elimination via proteasomal degradation of a synaptic scaffold PSD-95. Cell. 2012;151(7):1581-1594. doi:10.1016/j.cell.2012.11.040

Mir S, Sen T, Sen N. Cytokine-induced GAPDH sulfhydration affects PSD95 degradation and memory. Mol Cell. 2014;56(6):786-795. doi:10.1016/j.molcel.2014.10.019

Li L, Cao Y, Wu H, Ye X, Zhu Z, Xing G, Shen C, Barik A, Zhang B, Xie X, Zhi W, Gan L, Su H, Xiong WC, Mei L. Enzymatic Activity of the Scaffold Protein Rapsyn for Synapse Formation. Neuron. 2016;92(5):1007-1019. doi:10.1016/j.neuron.2016.10.023

Xing G, Jing H, Zhang L, Cao Y, Li L, Zhao K, Dong Z, Chen W, Wang H, Cao R, Xiong WC, Mei L. A mechanism in agrin signaling revealed by a prevalent Rapsyn mutation in congenital myasthenic syndrome. Elife. 2019;8:e49180. Published 2019 Sep 24. doi:10.7554/eLife.49180

Chen A, Bai L, Zhong K, Shu X, Wang A, Xiao Y, Zhang K, Shen C. APC2CDH1 negatively regulates agrin signaling by promoting the ubiquitination and proteolytic degradation of DOK7. FASEB J. 2020;34(9):12009-12023. doi:10.1096/fj.202000485R

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Sharma G, Banerjee S. Activity-regulated E3 ubiquitin ligase TRIM47 modulates excitatory synapse development. Front Mol Neurosci. 2022;15:943980. Published 2022 Sep 21. doi:10.3389/fnmol.2022.943980

Ma Q, Ruan H, Peng L, Zhang M, Gack MU, Yao WD. Proteasome-independent polyubiquitin linkage regulates synapse scaffolding, efficacy, and plasticity. Proc Natl Acad Sci U S A. 2017;114(41):E8760-E8769. doi:10.1073/pnas.1620153114

Bianchetta MJ, Lam TT, Jones SN, Morabito MA. Cyclin-dependent kinase 5 regulates PSD-95 ubiquitination in neurons. J Neurosci. 2011;31(33):12029-12035. doi:10.1523/JNEUROSCI.2388-11.2011

Chapter V

Conclusion

It is apparent through the evidence presented and the growing field of study that understanding the function of Nedd4-1 in the neuron is critical to learning more about neuronal function as a whole. An extensive literature review illustrates the wide diversity of biological processes Nedd4-1 is involved in and how its dysregulation may lead to a number of neurological disorders. This review elucidates Nedd4-1's deep entanglement into the complex web of molecular pathways working together in the neuron to not only maintain health but allow for dynamic changes in neuronal function, while simultaneously shinning a light on all we do not yet know about the ligase and its role in the neuron. Luckily, Nedd4-1's subcellular localization is tightly regulated by the neuron. Exploitation of this feature of the E3 ligase paired with time-lapse imaging and spatial proteomics techniques has allowed us to expand what is known about Nedd4-1 function, the biological pathways it interacts in, and the activity dependent rules that drive its translocation. Together this data will provide a platform to further unravel the mysteries of Nedd4-1 function in the central nervous system and the broader molecular mechanisms underpinning synapse plasticity and cognition.

A. Uncovering the rules regulating Nedd4-1 trafficking in the neuron

It is important for eukaryotic cells to regulate the subcellular localization of their protein network. Different cellular compartments have different chemical environments and protein makeups, so a protein's functional capabilities can be closely tied to their spatial localization. This is especially true in neuronal cell types that have highly adaptive demands

and long processes. Neurons feature complex cytoskeletal machinery and protein transport networks for this very reason. The E3 ligase, Nedd4-1 is no different in its need to regulate its subcellular localization. The ligase's C2 domain allows it to bind Ca^{2+} and phospholipid membranes to translocate in the neuron in an activity dependent manner. This feature of the ligase allows it to interact in numerous downstream pathways (motor development, receptor trafficking, neurodegenerative disease progression, etc.) making it vital to neuronal cell health and function. The more we can uncover about how Nedd4-1 is trafficked in the neuron, the more we can understand about its function, its finely regulated interactions, and the molecular mechanism of biological processes such as synaptic plasticity.

E3 ligase trafficking has been difficult to study seeing as not many ligases have been shown to translocate in an activity dependent manner. In addition, many of the traditional techniques for studying protein localization fail to include temporal information that allows one to describe the changing dynamics of protein trafficking. In this study, we developed a time-lapse imaging paradigm to observe real time translocation of Nedd4-1 in dissociated hippocampal neurons. This spatial-temporal information allowed us to define distinct trafficking rules for different glutamatergic receptor stimulations. We now know that Nedd4-1 subcellular trafficking is being finely regulated in the neuron and can use this time-lapse imaging paradigm to further probe questions about Nedd4-1 trafficking.

Future experiments will combine live imaging with single synapse activation techniques such as photo-activated glutamate uncaging to understand the rules regulating Nedd4-1 trafficking on a local vs a global scale (Goo MS. et al, 2017). Pharmacology and electrophysiology could be used to recapitulate various known plasticity paradigms to define

the rules regulating trafficking of Nedd4-1 under these conditions. It is known that the C2 domain and HECT domain of Nedd4-1 have a transient affinity for each other that allow it to exist in two states: active and inactive (autoinhibition). (Persaud A. et al, 2014) shows that phosphorylation of Nedd4-1 in the C2 and HECT domain may disrupt the binding affinity of the two domains and shift Nedd4-1 into a more active state. Genetic manipulation of Nedd4-1 to create phosphomimetic and phosphor-dead point mutations combined with live time-lapse imaging would allow us to ask questions about how posttranslational modifications of Nedd4-1 affect its translocation. Lastly, we could use mRNA imaging techniques such as RNA fluorescent in situ hybridization (FISH) to uncover the subcellular localization of Nedd4-1 mRNA in relation to live protein trafficking dynamics. These are just a few of the questions this time-lapse imaging platform will be able to answer. With the growing technology, the ability to live imaged Nedd4-1 and possibly other E3 ligases will play a huge role in elucidating these proteins function in the neuron.

B. Re-imaging Nedd4-1's role in protein homeostasis

Protein homeostasis (proteostasis) is the regulation of the relative abundances of all the proteins in a cell and can be broken into three arms: synthesis, maintenance, and degradation. The degradation of proteins in the cell is commonly mediated through the ubiquitin proteasome system (UPS), the final step being ubiquitination of proteins targeted for degradation by an E3 ligase. Because of this, E3 ligase's have been well studied for the essential role in proteostasis and therefore neuronal cell function. Up until this point though, a majority of the field studies E3 ligases only in the context of degradation and their ability to

regulate the removal of proteins from various subcellular compartments and biological pathways.

In this study, we set out to characterize the interactome of the E3 ligase, Nedd4-1. Nedd4-1 is a well-studied ligase that has been implicated in a number of molecular pathways (synapse plasticity, axon outgrowth, motor development, etc.) and the pathophysiology of a number of neurological diseases (Alzheimer's disease, Parkinson's disease, Schizophrenia, etc.). Understanding this ligase's network of interacting proteins could only further uncover its role in the neuron. To our surprise, we find that Nedd4-1 is highly associated with spliceosome complex components and mRNA processing. There is very little research showing any E3 ligase interacting with the mRNA processing pathway. This data introduces an idea that E3 ligase may participate in regulating synthesis as well as degradation in the proteostasis cycle. It could be possible that Nedd4-1 pumps the breaks on synthesis by degrading spliceosome components to maintain protein levels until neuronal demands change. Then by removing Nedd4-1 inhibition on synthesis and targeting the ligase to degradation in a particular cellular compartment you could see massive turnover and refreshing of proteins at that site. This is an attractive theory, but it does not take into account the great diversity of ubiquitin signaling. The amazing thing about ubiquitin molecules is that they can be modified in numerous ways (monoubiquitination, polyubiquitination, posttranslational modifications – phosphorylation, acetylation, SUMOylation) which allow for targeting of a substrate to a myriad of downstream pathways. Future experiments could utilize techniques to quantify translation and/or mRNA export from the nucleus in combination with genetic and pharmacological Nedd4-1 knockdown models to begin to understand Nedd4-1's role in

protein synthesis. Together these data could begin to help us in re-imaging Nedd4-1's place in protein homeostasis and neuronal function as a whole.

C. Exploring a functional role for NMDA recruitment of Nedd4-1 to the synapse

Synaptic plasticity is the ability of a neuron to modulate the strength of a single synapse. Synaptic strength can be almost directly correlated to the number of neurotransmitter receptors present at the surface to receive signals from pre-synaptic terminals. In order to understand synaptic plasticity, the field has had to explore how receptors are inserted and removed from the synapse. Two of the most well studied receptors at the synapse are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor, and the N-methyl-D-aspartate (NMDA) receptor. The number of AMPA receptors at the synapse is typically considered an accurate measure of synaptic strength due to the receptor's kinetics allowing them to mediate a majority of the fast-acting activity at the synapse. NMDA receptors are much more regarded for their high permeability to calcium and diversity of subunit composition, both of which allow them to mediate a variety of downstream pathways including various plasticity paradigms. Uncovering the properties and trafficking dynamics of both receptors will be essential to understanding synapse plasticity in the brain.

Our lab was this first to show that ubiquitination of the GluA1 subunit of AMPA receptors (AMPARs) by Nedd4-1 was important to the internalization and degradation of AMPARs (Schwarz LA. et al, 2010). This placed Nedd4-1 in an essential role for the removal

of AMPARs from the surface and as an important player in synaptic modulation. We also showed that AMPAR activation caused Nedd4-1 to aggregate in puncta formation and redistribute in the neuron, theoretically to ubiquitinate AMPARs (Scudder SL. et al, 2014). Surprisingly, none of our previous studies show NMDA treatment affecting the redistribution of Nedd4-1 in any manner despite NMDA's well described role in synaptic plasticity and AMPAR removal. In this study, we provide ample evidence of Nedd4-1 playing a role in NMDA receptor (NMDAR) mediated synaptic regulation. We see NMDA induced enrichment of Nedd4-1 with synaptic organizational proteins in our spatial proteomics. This is supported by increased colocalization with synaptic marker, PSD-95, in our fluorescent imaging and dysregulation of synaptic function when inhibiting Nedd4-1 activity in our electrophysiology. We also see that pharmacological inhibition of Nedd4-1 activity blocks NMDA induced reduction of surface GluA1 but not GluA2 containing AMPARs with our biochemical assays. It is apparent that Nedd4-1 plays an important role in NMDAR's ability to regulate synaptic function and organization. Future experiments will be able to further ask questions about the specific rules regulating Nedd4-1 role at the synapse such as: What regulators downstream of NMDA receptor activation may be modifying Nedd4-1 activity and recruitment to the synapse? How do posttranslational modifications of the AMPARs affect Nedd4-1's trafficking and function at the synapse? What do Nedd4-1's possible interactions with other synaptic proteins mean for the Nedd4-1 regulation of synaptic function? Expanding technology will allow us to answer some of these questions and gain a better understanding of Nedd4-1's involvement in synaptic regulation as well as the molecular mechanisms underpinning cognition in the brain.

D. Mapping Nedd4-1's participation in the pathophysiology of neurological disorders

E3 ligases have been implicated in neurological disease mechanisms in the field for a long time. Because of their critical role in protein homeostasis E3 ligase dysregulation can lead to a number of neurological disorders. Nedd4-1 in particular has been linked to neurodegenerative diseases, psychiatric disorders, cancer, and many other neuronal diseases. In this study, we set out to define a comprehensive Nedd4-1 interactome. We then took this interactome and performed an extensive literature search on each enriched term to identify the disease relevance of Nedd4-1's interactome. This comprehensive study will be able to serve as a resource for other labs as well as for further experimentation by uncovering tens of novel interacting pathways where Nedd4-1 could play a role in the pathophysiology of any number of diseases. Future experiments will begin by confirming Nedd4-1's interaction with novel enriched interaction partners. This would allow us to use the diversity of tools developed by our lab to modulate Nedd4-1 expression and function genetically, virally, and pharmacologically in combination with a variety of neurological disease models to answer questions about Nedd4-1's role in the pathophysiology of those disorders. This data will serve as a library of possibilities that hopefully informs various new studies uncovering Nedd4-1's role in the central nervous system.

H. References

Goo MS, Sancho L, Slepak N, Boassa D, Deerinck TJ, Ellisman MH, Bloodgood BL, Patrick GN. Activity-dependent trafficking of lysosomes in dendrites and dendritic spines. J Cell Biol. 2017;216(8):2499-2513. doi:10.1083/jcb.201704068

Persaud A, Alberts P, Mari S, Tong J, Murchie R, Maspero E, Safi F, Moran MF, Polo S, Rotin D. Tyrosine phosphorylation of NEDD4 activates its ubiquitin ligase activity. Sci Signal. 2014;7(346):ra95. Published 2014 Oct 7. doi:10.1126/scisignal.2005290

Schwarz LA, Hall BJ, Patrick GN. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. J Neurosci. 2010;30(49):16718-16729. doi:10.1523/JNEUROSCI.3686-10.2010

Scudder SL, Goo MS, Cartier AE, Molteni A, Schwarz LA, Wright R, Patrick GN. Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. J Neurosci. 2014;34(50):16637-16649. doi:10.1523/JNEUROSCI.2452-14.2014