Stony Brook University

The Intracellular Cell Biology of Ionotropic Glutamate Receptors

A Thesis Presented

by

Noele Doreen Certain

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

December 2017

Stony Brook University

The Graduate School

Noele Doreen Certain

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Lonnie P. Wollmuth, Ph.D., Professor
Department of Neurobiology and Behavior

Helen Hsieh, M.D., Ph.D., Assistant Professor of Surgery
Department of Surgery, Division of Pediatric Surgery

This thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Thesis

The Intracellular Cell Biology of Ionotropic Glutamate Receptors

by

Noele Doreen Certain

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

2017

AMPARs play an essential role in fast excitatory neurotransmission. Before native AMPARs can function at the synapse they must be processed intracellularly. These ion channels are synthesized and assembled into multimeric complexes, shuttled in a regulated fashion between intracellular compartments and are finally inserted into the membrane to carry out their function. Important binding partners known as auxiliary subunits associate with AMPARs at the synapse, but also during assembly and trafficking. The presence of auxiliary subunits in AMPAR complexes at the membrane has been demonstrated through electrophysiological studies, showing effects on the gating and pharmacology of AMPARs. The localization of AMPAR-auxiliary subunits complexes in cellular compartments has not been well characterized, and the stoichiometry of these auxiliary subunits for appropriate function is not known. The main objective is to further characterize auxiliary subunits beyond their effects on AMPAR gating at the synapse. Cerebellar Granule Neurons were to be used as the native environment to study AMPAR-auxiliary complexes to address both the localization of these complexes and the stoichiometry of auxiliary subunits. The prediction is that auxiliary subunits complex with AMPARs in a compartment-specific manner and this allows for the appropriate processing of AMPARs.

Dedication

I dedicate this work to my family and friends who have supported my every dream. And to George A. Whitman, Isabella Grovenstine Mayhenn and George Shammis who have passed on, but continue to inspire me.

Table of Contents

List of Figures

List of Abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATD	amino terminal domain
BN-PAGE	blue native polyacrylamide gel electrophoresis
CGN	cerebellar granule neurons
CI	Calcium impermeable
CNIH	cornichons
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CP	Calcium permeable
Cryo-EM	cryo-electron microscopy
CTD	carboxyl terminal domain
DIV	days in vitro
DMEM	dulbecco's modified eagle media
DNA	deoxyribonucleic acid
dsRED	*discosoma sp*. Red
ER	endoplasmic reticulum
FRET	förster resonance energy transfer
GFP	green fluorescent protein
GSG1-L	germ cell specific gene 1-like
HEK 293	human embryonic kidney 293
HRP	Horseradish peroxidase
ICC	immunocytochemistry
iGluR	ionotropic glutamate receptors
IRES	internal ribosome entry site

KO	knockout
LBD	ligand binding domain
LTD	long-term depression
LTP	long-term potentiation
NMDAR	N-methyl-D-aspartate receptor
P	postnatal day
PBS	phosphate buffered saline
PPI	protein-protein interactions
PSD-95	postsynaptic density protein
Q	glutamine
R	arginine
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
SAP	synapse associated proteins
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TARP	transmembrane AMPAR regulator protein
TBS-T	tris-buffered saline -tween 20
TMD	transmembrane domain

Acknowledgments

I would like to thank my advisor, Dr. Lonnie Wollmuth, for him taking a chance on me. His guidance and teachings have been instrumental in my success and understanding of research.

I would like to thank Dr. Helen Hsieh for mentoring and encouraging me to continue to push forward when it seems that everything else has failed.

I would like to thank Diane Henry-Vanisko, for her support throughout my start in the Wollmuth lab. You are an invaluable part of the lab without your wisdom, I would not succeed.

I would like to thank the Wollmuth Lab, past and current graduate and undergraduate students who have supported and encouraged me along this journey. Especially Gabrielle Moody, Johansen Amin, Kelvin Chen, Alex Baez, Camillo Rafael Ferrer Fierro, Aaron Gochman, Ayman Haider and Quan “Alfred” Gan.

I would like to thank the Talmage Lab and Ge Lab for their support and advice for developing the neuronal cultures and transfection protocol.

I would like to thank all my family and friends for the continued support and appreciating my dedication to my career. Especially my sister Nochelle who has always pushed me to triumph over any adversity.

Lastly, I would like to thank Jonathan Whitman for his unwavering support, love and faith in me. Without him, none of this would be possible.

Introduction

The central nervous system is an intricate network of neurons that coordinate signals and propagates responses to various targets within an organism. The structures where the cell-to-cell communication occurs between neurons are called synapses. These include chemical synapses defined by a chemical molecule or neurotransmitter being released by a presynaptic cell and acting on specific receptors of the postsynaptic cell to cause the propagation of the signal (Figure 1) (Purves et al., 2003). Chemical synapses can be further divided into excitatory and inhibitory subtypes, which together play crucial roles in the processing of information in the brain. Postsynaptic neurons can be excitatory or inhibitory depending on ion permeability of the ion channel activated by the neurotransmitter (Purves et al., 2003). An inhibitory synapse decreases the activity of a postsynaptic neuron, whereas excitatory synapses increase the probability of postsynaptic activity (Purves et al., 2003). The total neural population consists of approximately ~80% excitatory neurons regardless of the species, and will be further discussed due to the overwhelming importance within a functional synapse (DeFelipe et al., 2002).

Excitatory Synaptic Transmission

The physiology of a synapse depends on two components: the type of neurotransmitter released and the corresponding receptor at the postsynaptic membrane. Nearly all excitatory synaptic transmission in the central nervous system involves the pre-synaptic release of the neurotransmitter glutamate (Traynelis et al., 2010). Upon release, glutamate activates post-synaptic ligand-gated ion channels called iGluRs. Two major postsynaptic subtypes are AMPARs and NMDARs. All ionotropic glutamate receptors are non-selectively permeable to cations, allowing the passage of sodium, potassium and in some cases calcium (Purves et al., 2003). NMDARs demonstrate slow gating kinetics producing slow and long-lasting ionic currents, while AMPARs mediate rapid and brief currents at the synapse (Purves et al., 2003). The fast signaling of AMPARs also influence the behavior of neighboring NMDARs at the postsynaptic membrane (Greger et al., 2017). Both iGluRs are functionally distinct at an excitatory synapse and are important to synaptic function.

Perturbation of synaptic physiology due to dysfunction or dysregulation has been implicated in various neurological disorders and neurodegenerative diseases. For example, the protein amyloid-beta closely associated with the neurogenerative disease, Alzheimer's, can drive synaptic depression via endocytosis of AMPARs and has been linked to memory impairment

(Hsieh et al., 2006; Shankar et al., 2008). Synaptic function is necessary in maintaining sufficient and accurate communication within the central nervous system. A loss of synaptic regulation can lead to the disruption of synapses, and has been intimately linked to neurological and neurodegenerative diseases. Therefore, it is necessary to focus on the mechanisms that underlie the normal physiology of a synapse.

Synaptic Plasticity

Neural plasticity is the capacity of the nervous system to change in response to stimuli, most notably during development or rewiring of the neuronal circuits (Purves et al., 2003). The modulation of the functional proteins directly influences the formation and plasticity of a chemical synapse (Spronsen & Hoogenraad, 2010). The plasticity of the synapse is directly linked to changes in gene expression and post-translation modifications of synaptic proteins. The expression and modulation of synaptic proteins, such as iGluRs directly regulates synaptic transmission within a neural circuit.

At the molecular level, the sensitivity of an individual synapse to a stimulus is modulated by regulating synaptic proteins. The long-term changes occurring at a synapse are responsible for brain function, such as memory and learning (Purves et al., 2003). LTP is a long-lasting strengthening of synaptic transmission, while weakening of synaptic activity is defined as LTD (Purves et al., 2003). LTP and LTD are changes occurring at individual synapses produced by cellular and molecular mechanisms that regulate synaptic proteins. In an excitatory synapse, the presence of NMDARs are important for the induction of LTP, while insertion of AMPARs are necessary for the maintenance of the LTP (Purves et al., 2003). Loss of AMPARs at the synapse leads to LTD and decreased synaptic efficacy (Purves et al., 2003). The expression and insertion of these receptors are crucial for development of the synaptic plasticity (Greger et al., 2017) The ability to modulate individual synapses demonstrates how the nervous system can precisely modify neuronal circuits spatially and temporally.

The plasticity of the postsynaptic membrane is influenced by synchronized expression and modulation of AMPARs. Synaptic plasticity is not simply regulated by the expression of the gene. AMPARs are further coordinated by non-transient binding partners known as auxiliary subunits. The non-pore forming auxiliary subunits complex with AMPARs and govern the trafficking and/or channel properties, thus, they directly influence synaptic physiology (Yan & Tomita, 2012). Although the function of auxiliary subunits at the membrane has been previously defined, the role

of auxiliary subunits in the processing of AMPARs is still not well understood. The review will focus on the known assembly and trafficking of AMPARs, which is important to understand the intracellular regulation of AMPARs. In addition, the diversity of auxiliary subunits and the current knowledge of auxiliary subunit influence on pore-forming AMPARs will be reviewed.

AMPAR Assembly

AMPARs are assembled into tetrameric complexes from four subunits, GluA1-A4 (Figure 2). The expression and assembly of different AMPAR subunit mediates specific functional properties and affect the development of postsynaptic signaling. Subunit composition influences trafficking properties, gating kinetics, ion permeability of the receptor. AMPARs lacking GluA2 subunits, also known as GluA2-lacking receptors, are calcium permeable (Henley & Wilkinson, 2016). Widespread RNA editing of GluA2 subunits replaces Q607 with R, rendering GluA2-containing receptors calcium impermeable (Henley & Wilkinson, 2016). The change in calcium permeability of AMPARs is thought to contribute to synaptic plasticity (Henley & Wilkinson, 2016). The insertion of CP- or CI-AMPARs can vary with developmental stage, demonstrating AMPAR expression requires temporal regulation and can be subunit specific (Henley & Wilkinson, 2016).

According to Schwenk et al. (2014), brain regions including the hippocampus and cortex present GluA2 subunits as the predominant subunit (~45%), GluA1 and GluA3 at ~25% each, and GluA4 at ~6%. In contrast, the cerebellum region is dominated by GluA4 subunits (64%) (Schwenk et al., 2014). Auxiliary subunits were also found to be in specific brain regions. The proteome study illustrated a diverse regional distribution of AMPAR subunits natively associated with auxiliary subunits. The results suggest a mechanism to coordinate distinct arrangements of AMPAR complexes in the brain (Schwenk et al., 2014).

Prior to insertion into the membrane, these receptors require proper assembly and processing before the mature receptor is trafficked to the cell surface to contribute to synaptic transmission (Figure 3). AMPARs are assembled in the ER and require ER export, all which are thought to be highly regulated (Henley & Wilkinson, 2016). Assembled AMPARs are subsequently trafficked to the Golgi network and transported out via specialized vesicles. Mature and assembled AMPARs are then directed to the synapse to carry out their biological function. A major part of AMPARs function is the contribution of AMPAR protein domains that aid in receptor assembly, ER exiting and display important functional RNA editing. Each AMPAR subunit

consists of an extracellular ATD, LBD, three membrane-spanning TMDs, a re-entrant loop, and an intracellular CTD shown in Figure 2. These modular structures will be defined in their contribution to AMPAR assembly and function.

Amino-Terminal Domain

Improved structural resolution has helped to clarify the AMPARs through x-ray crystallography (Sobolevsky et al., 2009) and Cryo-EM projecting a picture of the AMPAR structure (Zhao et al., 2016; Twomey et al., 2016). The Cryo-EM structure visualized the ATD involved in AMPAR subunit oligomerization due to conformational changes seen in the protein structure. This was also demonstrated *in vitro*, purified ATDs possessed a relatively high affinity for each other, proposing a crucial role in the initial dimerization process (Kumar et al., 2009; Rossman et al., 2011; Mayer, 2011). The AMPAR ATD was recently proposed to be involved in AMPAR anchoring at the synapse and that LTP is dependent on the ATD (Watson et al., 2017). At the same dimer stage, the LBD domains are also proposed to organize into dimers, leading to the term a dimers-of-dimers (Figure 4) (Gan et al., 2015). Through various methods, the AMPAR structure is suggested to form a dimer-dimer intermediate based on single-particle EM, size-exclusion chromatography and BN-PAGE (Shanks et al., 2010; Salussolia et al., 2013). The localization of AMPAR dimer reserve pools, could suggest a region responsible for subunit selection prior to the receptor exiting the ER.

Ligand-Binding Domain

The LBD is most notably important for the binding of agonist and forming an interface leading to ion channel current (Traynelis et al., 2010). The structural resolution in Cryo-EM further demonstrates the LBD's significance to influence the activation of AMPARs through conformational changes known as the 'gating cycle' (Meyerson et al., 2016). The LBD is also functionally relevant suggested by RNA editing sites of the LBD sequence that impact the subunit assembly (Greger et al., 2006; Penn et al., 2008). Surprisingly, the presence of the LBD was also found to be necessary and defined as an energetic barrier or 'LBD barrier' that influences the stability of the receptor (Gan et al., 2016). Alternative RNA editing of the LBD generates two transcripts of GluA1-4, either existing as a flip (i) or flop (o) variants (Penn et al., 2012; Salussolia & Wollmuth, 2012). The splice variant region is dependent on the inclusion or exclusion of exon 14 or exon 15, just before the M4 region in the mature transcript (Penn et al., 2012). An alteration of GluA1/GluA2 assembly is observed when either flip/flop variants is removed suggesting that

alternative splicing in the LBD may be involved in AMPAR assembly (Penn et al., 2012). The presence of flip/flop splicing has also been shown to influence interactions with auxiliary subunits (Semenov et al., 2012).

Transmembrane Domain

Besides the contribution of the LBD and ATD, the TMD also influences the assembly of AMPARs. The TMD is composed of three transmembrane helical domains (M1, M3, M4) and a membrane re-entrant loop, M2 (Traynelis et al., 2010). The M2 loop integrates with other subunit M2 domains to line the inner structure and enter the cytoplasmic side of the membrane shown in Figure 2 (Traynelis et al., 2010). The M2 segment forms the inner channel pore narrowing at the base of the channel (Traynelis et al., 2010). Constriction at the opening of the channel pore, presumably forming a gate that regulates the flux of ions (Traynelis et al., 2010). The previously discussed RNA editing of the Q607R site is within the M2 sequence. This change at the apex of the pore loop eliminates calcium permeability and decreases physiological channel blocks (Greger et al., 2003). The RNA editing creates a major distinction between GluA2-lacking or GluA2-containing receptors. In addition, the Q/R site edit is prevalent in the majority of GluA2 RNA (Traynelis et al., 2010). The edited site of the GluA2 subunits showed increased dwell time in the ER, overall leading to increased availability and favoring the insertion of GluA2(R) into AMPAR tetramers (Greger et al., 2003). Overall the editing of the Q/R site directly influences AMPAR tetramerization.

The M3 transmembrane helix is connected to the LBD region via linkers of amino acids, thought to be involved in the activation of gating (Greger et al., 2017). Mutations in the M3 helices, have been shown to impact the ionic current and modulation of partial agonist binding efficacy (Moore et al., 2013). Intercalated between neighboring subunits, the M1-M3 domains form the structural core of the channel depicted in Figure 4. Furthermore, Gan et al. (2016) has demonstrated that the TMD is necessary for AMPAR tetramerization and exhibits a drastic influence on proper assembly compared to the other modular domains. Specifically, the M4 segment of the TMD has been repeatedly shown to be required for tetramerization of AMPARs (Salussolia et al., 2013; Gan et al., 2015).

The M4 uniquely interfaces with core M1-M3 domains of the neighboring AMPAR subunit (Gan et al., 2015). In addition, the M4 segment possesses a highly conserved ‘VLGAVE’ motif and manipulation of this sequence led to inefficient tetramerization (Gan et al., 2016). The M4

transmembrane helices have become an interesting component of the transmembrane region. In all eukaryotic iGluR subunits the M4 segment is present compared to prokaryotic iGluR subunits, demonstrating evolutionary relevance (Salussolia et al., 2013). The M4 has been demonstrated to be critical to tetramerization of AMPARs for successful surface expression (Salussolia et al., 2013; Gan et al., 2016; Amin et al., 2017).

Carboxyl-Terminal Domain

AMPARs have a cytoplasmic CTD that extend to different lengths depending on the subunit, and displays alternative splicing events (Greger et al., 2017). The function of the CTD has been implicated in the trafficking and localization of AMPARs. Functional studies have uncovered involvement in the tight regulation by phosphorylation and interactions with post-synaptic proteins PSD-95, SAP proteins e.g. (Kristensen et al., 2011; Anggono & Huganir, 2012). It is known that the activity of the CTD effects AMPAR trafficking in a subunit composition dependent manner and indirectly synaptic plasticity (Gough, 2007). However, it is not known whether these modifications are directly altering protein-protein interactions that participation in AMPAR assembly or altering trafficking to the membrane. AMPAR modular domains contribute to the development of mature AMPAR complexes.

Auxiliary Subunits

All native AMPARs are complexed with auxiliary subunits, which modulate the assembly and forward trafficking of AMPARs. Therefore, when investigating the function of AMPARs in the brain, it is necessary to consider auxiliary subunit influence. Auxiliary subunits are critical in regulating the trafficking, gating and pharmacology of the AMPAR-type iGluRs. This unique aspect is an integral part of AMPAR biogenesis and leads to the expansion of AMPAR function at the synapse.

Auxiliary subunits are defined by four criteria distinguishing them from other AMPAR transient and/or scaffold proteins (MAGUKs, Shank e.g.) (Vessey & Karra, 2007). First, an auxiliary subunit is defined as a non-pore-forming subunit. Therefore, no auxiliary subunit can have channel activity when expressed alone. Second, the interactions between the pore-forming AMPAR subunit and the auxiliary subunit must be stable and direct. In defining the direct, interaction, the auxiliary subunit must directly bind to AMPAR domains to mediate control. As for the stability of the interaction, the auxiliary subunit needs to be able to sustain association with AMPARs in a native environment. Third, the interactions of auxiliary subunits must be able to

modify the channel properties and/or trafficking *in vivo* in heterologous cells, when co-expressed with AMPARs. Finally, the auxiliary subunits should be necessary for certain channel properties observed in native ion channels. This is necessary to distinguish the native auxiliary subunits compared to non-native proteins that can behave like native auxiliary subunits. (Yan & Tomita, 2012)

The discovery of auxiliary subunits has illuminated the mechanisms behind AMPAR modulation. Recently, structural studies have shown auxiliary subunits associate with the TMD, suggesting a mechanism of modulating AMPARs through its modular domain (Twomey et al., 2016; Zhao et al., 2016). The CTD has a highly variable sequence and display possible binding sites for proteins including auxiliary subunits. This feature may allow specific partnering of AMPARs to auxiliary subunits. Studies have shown auxiliary subunits preferentially interact with only long CTD containing AMPARs (GluA1 and GluA4) (Soto et al., 2009). Auxiliary subunits are diverse and are currently classified based on structure and homology. This investigation will focus on the comparison of auxiliary subunits: TARP γ-2, TARP γ-8, CNIH-2, CNIH-3 and GSG1-L (Figure 5). While the impact of γ-2 on trafficking and assembly of AMPARs is well known, auxiliary subunits like CNIHs and GSG1-L are not well understood.

Transmembrane AMPAR Regulatory Proteins

The first class of auxiliary proteins to be discovered and interact with AMPARs are TARPs (Chen et al., 2000; Vandenberghe et al., 2005). TARPs are classified into two subtypes of TARPs, Type I: γ-2, γ-3, γ-4, γ-8 and Type II: γ-5, γ-7. These distinctions are based on both the TARP protein influence on AMPARs and the presence of a PDZ binding domain in the TARP carboxyl-terminal tail (Yan & Tomita, 2012). Stargazin, also known as γ-2, was the first auxiliary subunit to be discovered and is very well characterized. TARPs are four transmembrane domain proteins that share homology with the subunits of voltage-gated calcium pore-forming channels (Figure 5) (Chen et al., 2000). However, when TARPs are expressed they do not form pore-forming channels and instead modulate the pore-forming AMPARs (Vandenberghe et al., 2005).

TARPs associate with AMPARs at the postsynaptic membrane and modulate receptor current and pharmacology. The association of TARPs in AMPAR complexes leads to changes in ion channel function including the activation time, the rate of deactivation and desensitization (Preil et al., 2005; Menuz et al., 2007). Besides, the involvement in gating of a channel, it has been established that TARPs play a role in trafficking to the plasma membrane. An invaluable clue of

TARPs' role in trafficking was observed in stargazer mice that lacked functional γ-2. The absence of γ-2 led to the loss of functional AMPARs in synapses of the cerebellar granule neurons (Chen et al., 2000; Vandenberghe et al., 2005). Further investigation found AMPAR surface expression could be easily rescued by the exogenous expression of type I TARPs only (Tomita et al., 2003). The functional study further revealed a compensatory role between type I TARPs and found a functional difference to type II TARPs (Tomita et al., 2003).

The type I TARPs are essential for the insertion and removal of AMPARs from the membrane and can contribute to the development of LTP and LTD (Vandenberghe et al., 2005; Ziff, 2007). The TARP-AMPAR interactions are suggested to start within the ER based on analysis of protein modifications of the AMPARs (Tomita et al., 2003). TARPs are proposed to act as non-canonical chaperones for AMPARs (Tomita et al., 2003). TARPs have also been shown to prefer AMPAR subunits, which influences the AMPAR complexes trafficking to the membrane (Menuz et al., 2008). Evidence supports that γ-2 promotes AMPAR exit from the ER, Golgi export and trafficking, and γ-8 are observed to be similar in promoting AMPAR expression at the membrane (Haering et al., 2014). In contrast, γ-2 promotes high-density AMPAR expression, while γ-8 is suggested to support basal level expression of AMPARs at the synapse (Rouach et al., 2016). Furthermore, the mechanism behind these type I TARPs' similarities and differences in AMPAR function has yet to be defined.

Cornichons

CNIHs consist of three transmembrane domains and influence the function of AMPARs (Figure 5) (Haering et al., 2014). CNIHs were first identified in the *Drosophila* species (Schwenk et al., 2009). CNIHs were later found in a mammalian brain proteome analysis as CNIH-2 and CNIH-3 (Schwenk et al., 2009). Co-assembly of CNIH proteins with AMPARs, promote surface expression and alter the channel gating of AMPARs (Schwenk et al., 2009). CNIHs is involved in the positive modulation of AMPAR trafficking, acting as ER cargo adaptors and enhance Golgi trafficking (Harmel et al., 2012). CNIHs co-expressed with AMPARs alter the glycosylation pattern of the receptor for preferential ER export (Harmel et al., 2012).

Herring et al. (2013) has shown that CNIH proteins selectively associate with AMPARs containing GluA1. In hippocampal neuronal cultures, the loss of CNIH proteins leads to the depletion of GluA1-containing receptors at the synapse (Herring et al., 2013). This study further suggested γ-8 was preventing CNIH association with specific AMPARs, directly mediating CNIHs

influence on AMPARs (Herring et al., 2013). CNIH-2 has also shown to competitively lessen the formation of AMPAR-TARP complexes, but favorable associates with AMPAR-TARP complexes that contain γ-8 (Gill et al., 2011). This functional evidence has displayed that CNIHs are, competing with certain TARPs, potentially due to similar or overlapping binding sites of the AMPAR complexes (Haering et al., 2014).

Germ Cell Specific Gene 1-Like

Recently identified auxiliary protein, GSG1-L was discovered using affinity purification and mass spectroscopy from AMPAR complexes isolated from rat brain (Shanks et al., 2012). GSG1-L shares homology to the claudin family proteins, which includes the TARPs (Haering et al., 2014). GSG1-L is predicted to consist of four transmembrane domains, but contains a distinctly longer loop adjacent to the amino-terminal region of the protein compared to TARPs (Figure 5) (Shanks et al., 2012). Expression of GSG1-L leads to modulation of AMPAR channel properties by decreasing the rate of deactivation and desensitization (Shanks et al., 2012). GSG1-L expression also demonstrated a suppressive action on AMPAR surface expression (Shanks et al., 2012). In recent functional studies, GSG1-L suppressed CP-AMPARs at the synapse and diminished calcium current flow through AMPARs (McGee et al., 2015). GSG1-L assumes the role of an inhibitory element, further strengthened by its overexpression reducing AMPAR presence at the postsynaptic membrane (Shanks et al., 2012). Genetic studies also showed in GSG1-L KO rats lead to an increase in AMPAR expression at the synapse (Shanks et al., 2012).

AMPAR-Auxiliary Subunit Complexes

Given the continued expansion of auxiliary subunits associated with AMPARs, it is necessary to understand the native architecture of AMPAR complexes and characterize the composition including auxiliary subunits. For AMPAR trafficking, assembly begins in the ER and receptor stoichiometry is a tightly regulated process that involves the influence of auxiliary subunits. CNIHs act as ER exporters and promote exit from the ER and Golgi, seen by the altering of the glycosylation pattern of the AMPAR complexes (Schwenk et al., 2009; Harmel et al., 2012). Many studies indirectly suggest TARPs also interacts with AMPARs at the ER, shown by changes in the glycosylation pattern of AMPARs (Tomita et al., 2003; Vandenberghe et al., 2005). Preliminary work in our lab has shown that CNIH impacts AMPAR tetramerization in the endoplasmic reticulum whereas TARPs do not. This suggested that CNIHs are localizing further upstream than that of TARPs, compared to what has been previously shown.

The native localization of AMPAR-auxiliary complexes during assembly and trafficking to the postsynaptic membrane has yet to be directly shown. The first aim is to characterize the localization of these AMPAR-auxiliary subunits in neurons. Reconstructing a native environment is necessary for investigating the intracellular processing of AMPARs. Endogenously expressed AMPARs and auxiliary subunits are distributed throughout the mammalian brain in various combinations, demonstrated by Schwenk et al. (2014). In the mouse cerebellum, GluA1, γ-8, CNIHs and GSG1-L are not endogenously expressed or express at relatively low levels (Schwenk et al., 2014). Therefore, CGNs will serve as background for studying GluA1, γ-8, CNIHs and GSGL-1. The optimal stoichiometry of auxiliary subunits also is unknown and its effect on the intracellular processing of AMPARs. The second aim is to define native stoichiometry of auxiliary subunits complexing with AMPARs. In the native environment, FRET will be used to quantify the number of auxiliary subunits such as γ-2 that natively associate with AMPARs. Investigating these features of auxiliary subunits may suggest a motive for why similar auxiliary subunits have a varying influence on AMPAR assembly and trafficking. Specifically, investigating the difference between γ-2 and γ-8 in the mammalian brain.

Overall, this study aims to investigate auxiliary subunits' ability to expand the functional diversity of AMPARs before insertion into the postsynaptic membrane. I hypothesize that auxiliary subunits interact with AMPARs in a compartment-specific manner and their interactions are critical for the robust trafficking of AMPARs in a native environment. We will also develop approaches to characterize auxiliary-AMPAR stoichiometry. The knowledge of composition and localization of AMPAR-auxiliary complexes is crucial to understanding synaptic function and its contribution to plasticity in the brain.

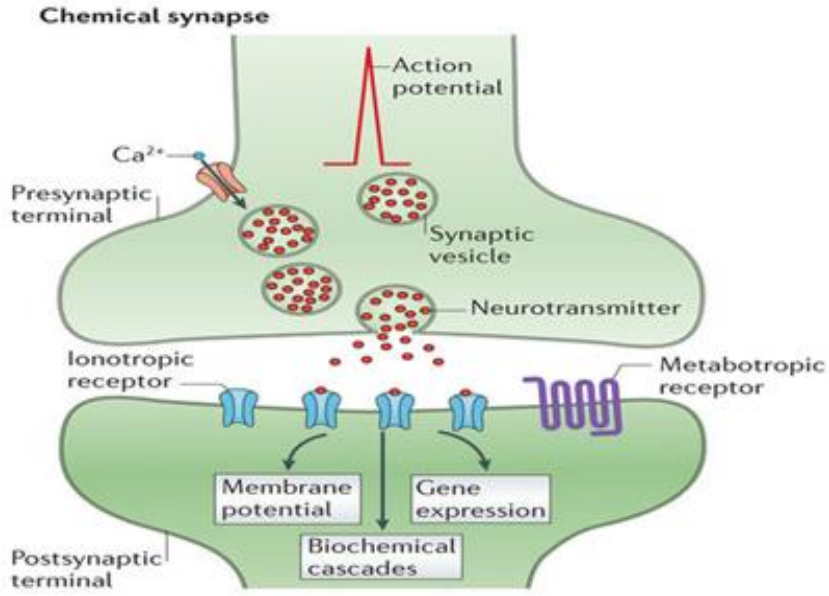


Figure 1. Chemical synapses feature no direct flow of current from the pre- to postsynaptic cell. Instead the postsynaptic current is generated by secretion of a neurotransmitter that acts on postsynaptic ligand-gated ion channels. Adapted from Pereda (2014).

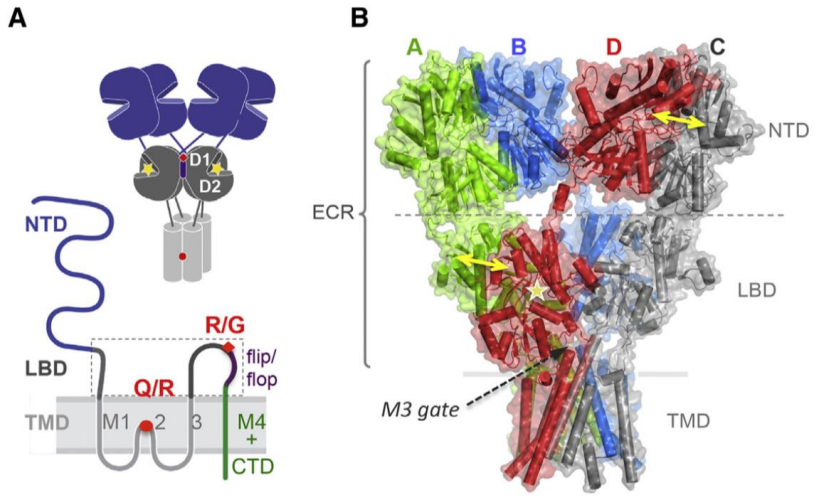


Figure 2. (A) The modular domains of AMPAR depicted, along with the topology of a single subunit containing each element that contribute to the AMPAR complex. Including the mRNA edit site in the M2 region, also the region of the flip/flop cassette above the M4 transmembrane region. Note: This is generic and does not show the differential between AMPAR subtypes. The stars represent where the agonist binds. (B) The x-ray crystal structure of the GluA2 homotetramer from PDB:3KG2 (Sobolevsky et al., 2009), where each polypeptide AMPAR subunit is distinctly colored. Adapted from Greger et al. (2017).

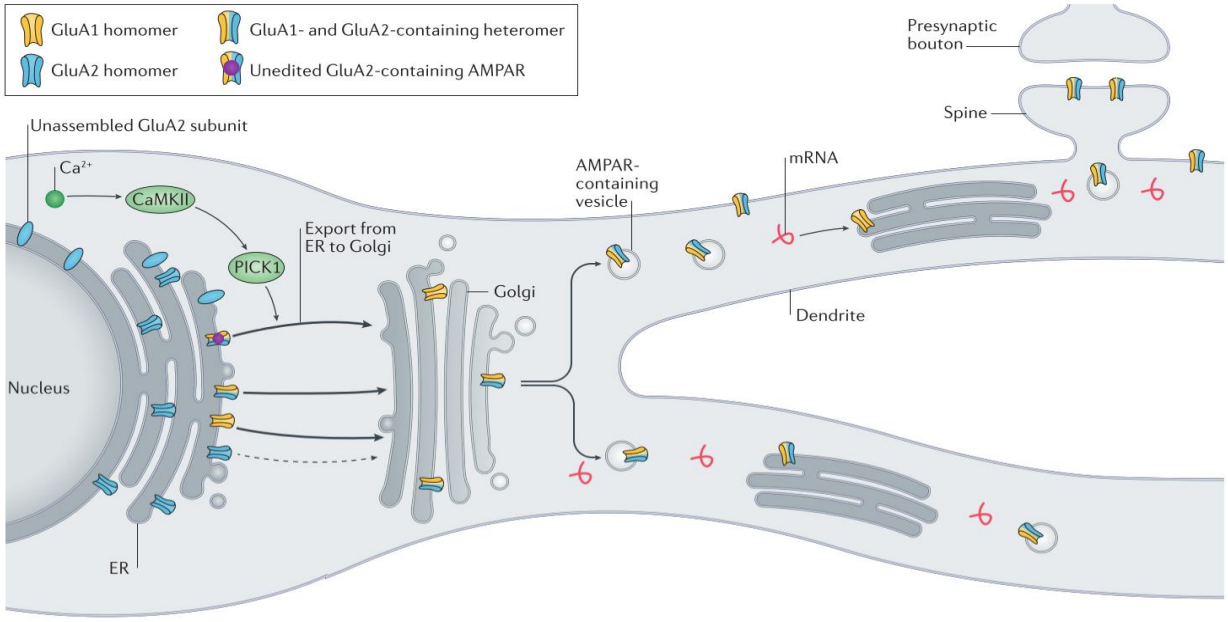


Figure 3. A diagram depicting AMPAR trafficking from the ER, through the Golgi network to postsynaptic synapses. Various subcellular organelles are involved in the assembly and trafficking of AMPARs. The subunit-specific AMPARs are assembled and exit the ER in a highly regulated fashion. Adapted from Henley & Wilkinson (2016).

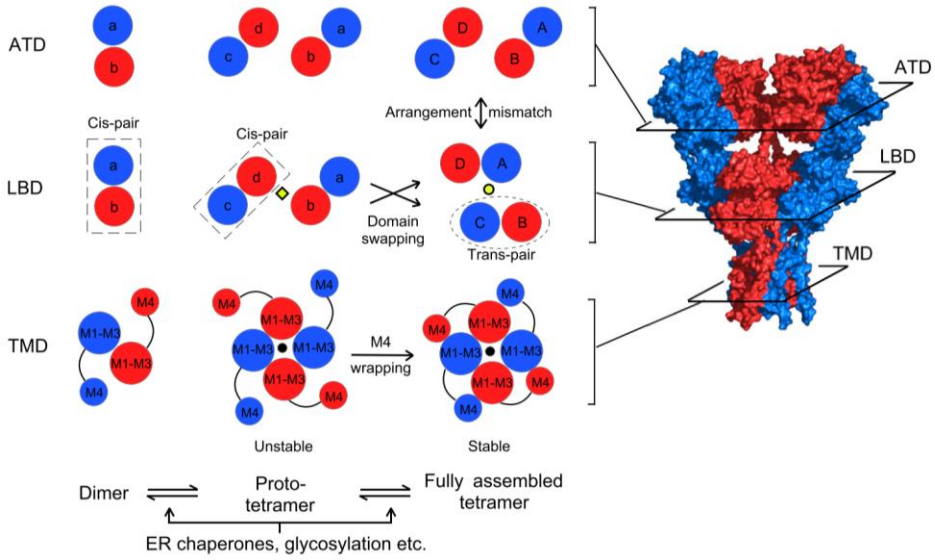


Figure 4. A diagram depicting assembly of the AMPAR modular domains. The three major events are the formation of the dimer driven by the ATD, the formation of an un-stable intermediate and the formation of the fully assembled receptor completed by the wrapping of the M4 domain and domain swapping event in the LBD. Adapted from Gan et al. (2015).

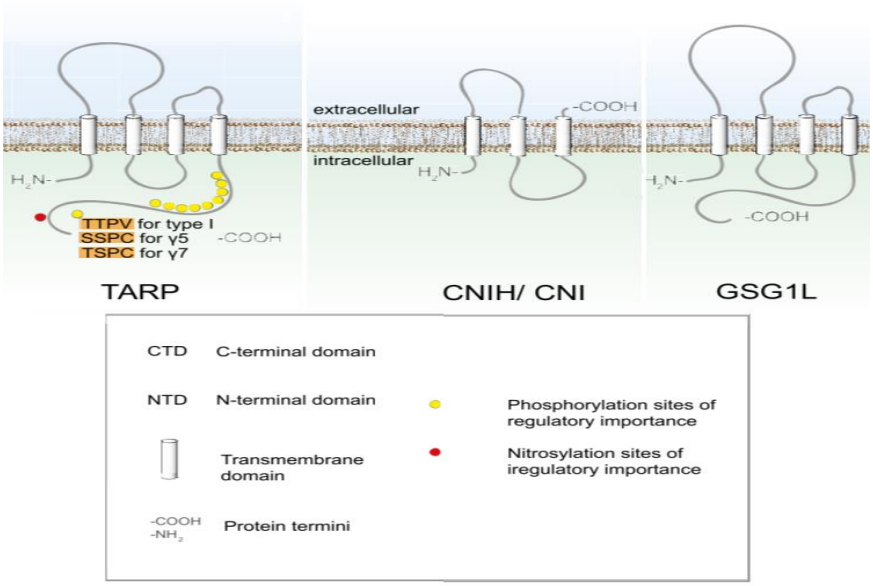


Figure 5. Schematic structure of TARPs, CNIH and GSG1-L. Displaying both their similarity and differences in structure. TARP and GSG1-L share homology to the Claudin protein family. Adapted from Haering et al. (2014).

Materials and Methods

Plasmid Constructs

Experiments were conducted with constructs GluA1 (*rattus noregicus*) all in the flip form: GluA1 (accession #P19490). AMPAR tandems provided by the Roger Nicoll Lab were used for transfection of rGluA1 flanked at the CTD by base pair repeats and fused to γ-2 along with IRES domain followed by dsRED (1:1 ratio).

Human Embryonic Kidney 293 Culture and Transfection

Wildtype and mutant AMPARs were expressed in HEK 293 cells. For 24 hours before transfection, HEK 293 cells were cultured on $3x10^5$ cells per 60 mm culture dishes in DMEM, supplemented with 10% FBS. HEK 293 cell were co-transfected with X-tremeGENE HP (Roche) at 60% confluency with 2 µg of cDNA constructs with pEGFP-C1(Clontech) at a ratio of 9:1, unless the construct contained a fluorescent tag no pEGFP-C1 was used. For HEK 293 cells transfected with AMPARs, 10µM CNQX (Sigma) a competitive antagonist was added to improve cell viability. After 48 hours, the cells were harvested for western blot analysis.

Cerebellar Granule Neuronal Cultures

Mouse CGNs were cultured from cerebella of P5-6 C57BL/6J pups through mechanical and enzymatic dissociation (trypsin 0.25% and DNase I 0.001%). The heterogenous cell lysate were subjected to a Percoll gradient separation to further purify the cerebellar granule neurons, from purkinje cells, glia cells and interneurons (Lee et al., 2009). Subsequently after Percoll gradient for purification of CGNs, the cells are manually counted and divided into aliquots of $4x10^6$-$5x10^6$ cells per test tube. The 6-well culture plates that are previously coated in low molecular weight poly-D-lysine (Sigma) overnight are rinsed and incubated with serum-free media at 37ºC in 5% CO_2.

Cerebellar Granule Neuron Nucleofection

After the Percoll gradient, the CGN suspension is centrifuged and the media is aspirated, and cells are resuspended in 100 µl transfection solution (Shayou Ge Lab). For transfection of cDNA, CGNs were nucleofected on DIV0 with either 2 micrograms (µg) cDNA or 2 µg pcDNA.GFP as a control using the program G-013, C-013, A023 following the Optimized Protocol for Primary Mouse

Neurons (Amaxa) using the Lonza Amaza Nucleofector™ 2b. The cells were subsequently plated after transfection on to the incubated plates, in Neurobasal medium (Gibco) supplemented with 10% fetal bovine serum, 100X penicillin-streptomycin, 100X GlutaMAX. Cytosine Arabionoside (1 uM) was added DIV1 to prevent glia cell proliferation. Cell cultures were examined through Olympus CkX41 inverted microscope and imaged using AccuScope camera on AccuView program.

Protein Harvest and Western Blot

The HEK 293 cultures were washed with cold 1X PBS, the cells were lysed and suspended I in RIPA buffer for whole cell lysate. The cells were agitated and incubated for 30 minutes at 4ºC and then centrifuged (Eppendorf Centrifuge 5417R) at 12,000 rpm for 20 minutes at 4ºC. The membrane proteins contained in the supernatant were mixed with Laemmli 2x buffer. The protein samples and PageRuler Plus Prestained Protein ladder were separated by a 5% SDS-PAGE gel run at 100V for 1 hour at room temperature. The proteins were transferred from the polyacrylamide gel to nitrocellulose membrane (0.45 mm) by wet transfer (BioRad) using 1X Tris-glycine buffer with the addition of methanol. The transfer of proteins to the membrane were confirmed with 2 % Ponceau S stain (Sigma) and then destained with TBS-T. The nitrocellulose membrane was blocked overnight with 5% non-fat milk protein in TBS (1x) at 4 ºC. The blocked membrane was incubated with anti-GluA1 (Millipore, MABN1116, Rabbit Monoclonal Antibody RH95) for 1 hour at room temperature under constant agitation and washed with TBS-T. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnologies, sc2030). The blot was developed using luminol reagent (Santa Cruz Biotechnologies, sc-2048) and exposed to chemiluminescent blue-sensitive film.

Results

Figure 6. Cerebellar Granule Neurons cultured in P5 pups (Nucleofected with p.EGFP-C1on DIV0). Imaged using Olympus CkX41 inverted microscope.

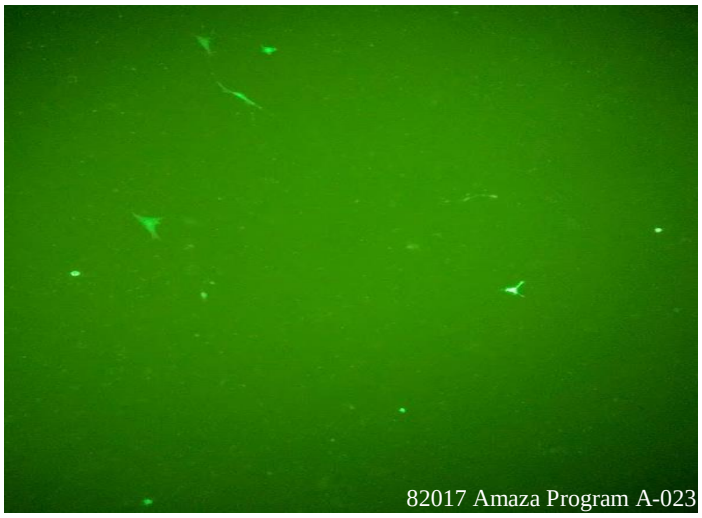


Figure 7. Cerebellar Granule Neurons cultured in P5 pups (Nucleofected with p.EGFP-C1 on DIV0). Imaged using Olympus CkX41 inverted microscope.

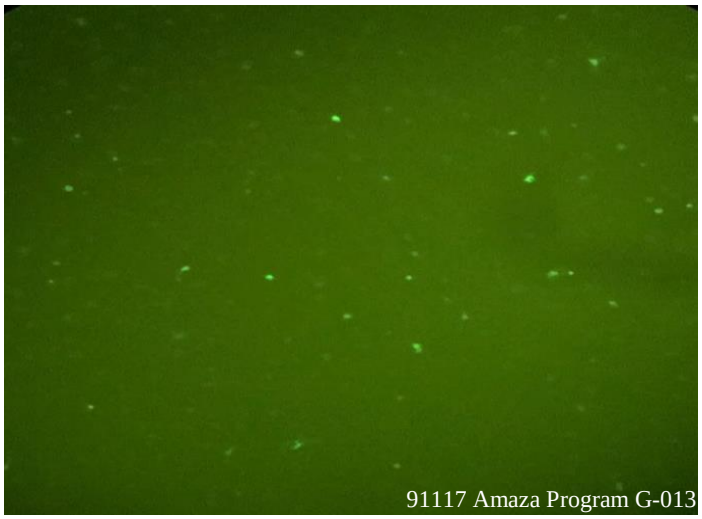


Figure 8. (a) HEK293 Cells (Co-transfected with GluA1.pRK7 and p.EGFP-C1) and (b) HEK293 Cells (Transfected with GluA1-γ-2-IRES-dsRed.p). Imaged using Olympus CkX41 inverted microscope.

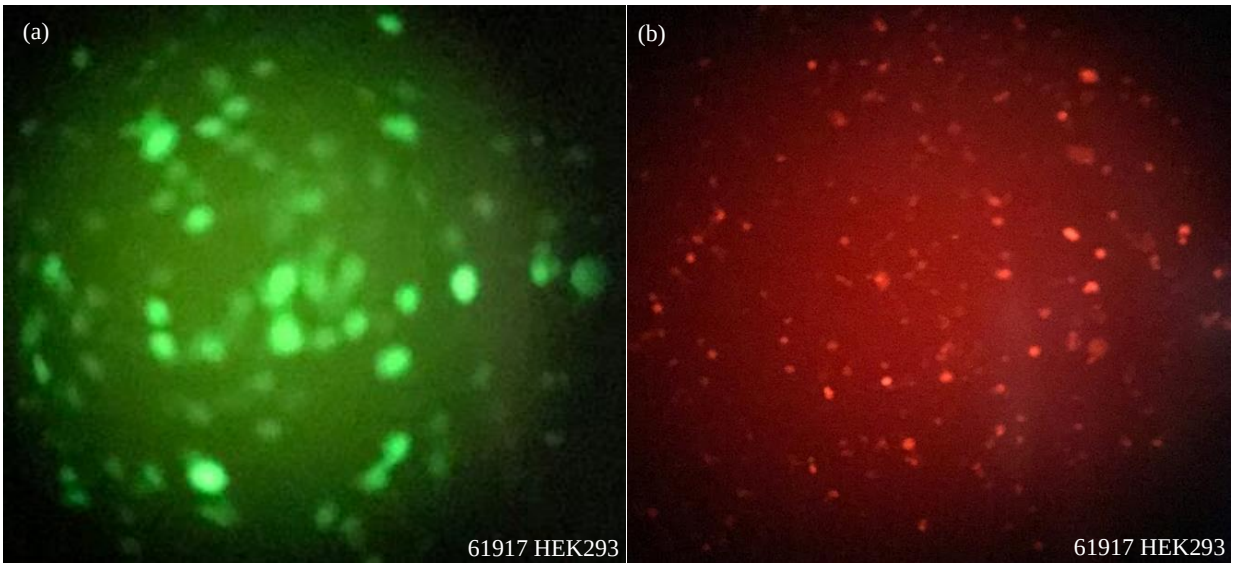
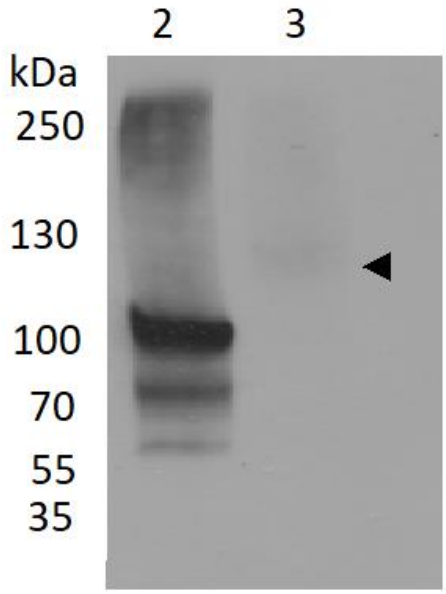


Figure 9. Western blot of transfected HEK 293 Cells (Harvested 48 hours post-transfection). Primary Antibody: Rabbit anti-GluR1 Secondary Antibody: goat anti-rabbit HRP conjugated. Lane 1 contains PagePlus Ruler (not shown), Lane 2 contains pRK.rGluA1, and Lane 3 contains pIRES.rGluA1-γ-2-IRES-dsRed respectively. Black arrow indicates weak band in Lane 3.



Discussion

Auxiliary subunits are necessary to regulate the intracellular processing and synaptic function of AMPARs. Previous studies have shown the importance of auxiliary subunits at the postsynaptic membrane, yet there is a lack of understanding of how auxiliary subunits participate in intracellular processing. Investigating in the native environment of AMPARs is necessary to understand auxiliary subunits role in AMPAR assembly and trafficking.

Nucleofection of Neuronal Cell Cultures

Nucleofection is an improved form of electroporation that uses an electric current to transfer DNA into the nucleus of a target cell (Karra et al., 2010). Nucleofection also provides a high transfection efficiency, necessary to biochemically isolate the proteins of interest for methods such as immunoblotting (Gartner et al., 2006; Karra et al., 2010). In Figure 6, nucleofected CGNs are indicated by GFP fluorescence showing a transfection efficiency of less than 2%. The low transfection of the CGNs demonstrated that the nucleofection process can heavily depend on the cell type and requires optimization of the protocol for higher transfection efficiencies. The process of nucleofection also presents a physical stress to the neurons. P5-P6 mice cerebellum were used because this time point contained the largest yield of CGNs according to Hatten et al. (1998) and Lee et al. (2009). Overall this improved the number of viable cells after the nucleofection process.

Figure 7 shows nucleofected CGNs containing GFP fluorescence after the protocol was continually optimized by increasing the cell count and repeated testing of several nucleofection programs on the Amaxa Nucleofector. The nucleofection programs are set to both alter the duration and intensity of the electrical current designed by Amaxa, for specific cell-types. The most effective Amaxa programs, which showed the largest number of GFP-positive CGNs were G-013 and C-013. However, the transfection efficiency was observed to be less than 5%. This result demonstrates the challenge of transfecting post-mitotic cells such as neurons, because they can be very resistant to the introduction and expression of exogenous constructs (Karra et al., 2010). Neuronal cultures are highly sensitive to physical stress, alterations in temperature, pH and changes in osmolarity, all of which can affect the transfection efficiency (Karra et al., 2010).

Nucleofection also presents limitations for when cell cultures can be transfected. For adherent cell cultures like CGNs they need to be nucleofected prior to plating. Neuronal cultures therefore need to be nucleofected DIV0. Nucleofecting neurons the day of dissociation does not allow cells to recover and can lead to more cell death. Overall, the nucleofection method needs to

be optimized further to increase the transfection efficiency to around 60-80% that several studies have observed (Gartner et al., 2006; Zietelhofer et al., 2009).

Western Blot Analysis of AMPAR Complexes

For the biochemical analysis of GluA1 and GluA1-γ-2 (tandem construct), SDS-PAGE and western blot were initially performed. HEK 293 cells were used because they are easily established and capable of large exogenous expression of proteins through simple transfection (Nishimura et al., 2000). The transfected HEK 293 are shown in Figure 8 indicated by either (a) GFP fluorescence or (b) dsRED fluorescence confirming successful transfection. The western blot in Figure 9 shows each sample processed and probed for GluA1. In lane 1, GluA1 is readily detected but forms multiple bands of various weights on the blot. The molecular weight for AMPAR assembly states is: ~700 kDa for tetramer, ~400 kDa for dimers, ~175 kDa for monomers (Reimers et al., 2011).

Figure 9 displays bands between 250 kDa to 55 kDa in lane 1, these results are not consistent with current analysis of AMPAR complexes. One reason for inconsistency of protein bands is using SDS-PAGE. This technique leads to denatured proteins, therefore the AMPAR complexes cannot easily be discriminated as monomeric, dimeric or tetrameric (Reimers et al., 2011). In Figure 9, for the GluA1 tandem construct (lane 3), shows a relatively low indistinct signal indicated by a black arrow. This result suggests the auxiliary subunit tandem is interfering with the antibody binding to the AMPAR. This analysis overall demonstrates limitations of western blot in isolating intact AMPAR complexes, however this is already well known and several have used BN-PAGE to isolate AMPAR complexes (Salussolia et al., 2013; Schwenk et al., 2014; Gan et al., 2016).

Future Directions and Alternative Methods

For recovery of native protein complexes, BN-PAGE is an ideal technique because it allows for native extraction of proteins while preserving protein structure and protein-protein interactions (Wittig et al., 2006). When using BN-PAGE for isolating AMPAR complexes this can reveal the auxiliary subunits complexed with the AMPAR in specific cellular compartments. The detection of auxiliary subunits also needs to be improved because they possess a relatively low molecular weight and cannot be detected in a gel shift. This can be accomplished by using a GFP-tagged auxiliary subunit to allow for the detection of auxiliary subunit presence. This also gives the ability to decipher between endogenous and exogenous auxiliary subunits.

For isolation of the AMPAR complexes in neurons, it is necessary to have a high percentage transfection efficiency for protein isolation. The nucleofection method requires a great deal of optimization shown by the inability to obtain above a 10% transfection of cells. Another alternative to performing nucleofection, would be viral transfection of CGNs. There are many advantages in using viral transfection, including a high transfection efficiency, stable genomic integration and presents a relatively low toxicity (Karra et al., 2010). Lentivirus is a retrovirus already used to transfect neuronal cell cultures, especially non-dividing cell types (Karra et al., 2010). One disadvantage is seen in the non-site-specific integration into the genome which could lead to mutagenesis, this can be corrected with a non-integrating lentivirus (Wanisch et al., 2009; Karra et al., 2010). Viral transfection will also allow the transfection of the neurons beyond DIV0, allowing for the neuronal cultures to become well established after culturing. Lentivirus is a better alternative for future experiments compared to the nucleofection method, and requires less optimization.

Subcellular fractionation is also needed to resolve the AMPAR complexes from specific compartments of the cells. To further the investigation, AMPARs complexed with auxiliary subunits need to be isolated from the ER, Golgi network, and plasma membrane. Preformed continuous iodixanol gradient is a common technique used to fractionate the Golgi network, ER and plasma membrane (Graham, 2002). Several studies have performed this technique in cell lines and were able to visualize modulation of ubiquitin-containing proteins from the Golgi network compared to the ER, along with protein turnover between different compartments (Yang et al., 1997; Zhang et al., 1998). Overall this method could allow for the visualization of AMPAR-auxiliary complexes for where they are occurring intracellularly.

An alternative method to subcellular fractionation and BN-PAGE analysis, is to perform immunocytochemistry (ICC) though it is less definitive. Immunocytochemistry involves the fixing of the cells and permeabilizing the membrane, for the antibodies to bind the proteins of interest (Ray et al., 2009). ICC can be used to probe for both AMPARs and auxiliary subunits concurrently while probing for specific cellular compartments (ER, Golgi network e.g.). A disadvantage of ICC is the occurrence of cross-talk between the different fluorophores on the secondary antibodies that are detected (Ray et al., 2009). To correct for crosstalk and obtain a higher resolution, confocal microscopy can be used. The advantages of using a confocal microscopy is the ability to label your

specimens with one or more fluorescent probes and the elimination ‘out of focus’ flare, commonly a disadvantage in conventional widefield optical microscopy (Paddok et al., 2017).

A future approach to characterize the auxiliary subunit stoichiometry will be the use of FRET. The employment of FRET has become a valuable tool in analyzing PPIs. FRET is a distance-dependent interaction where the donor and acceptor molecules must be in-close proximity and can be implied in vivo (Udea et al., 2013). Beyond the ability to measure protein-protein interactions, FRET offers an opportunity to quantitatively assay the stoichiometry of signaling in living cells. In Ben-Johny et al. (2016), FRET is used to determine the stoichiometry of calmodulin, a calcium binding protein, and its ability to bind to various voltage-gated calcium and sodium channels. Their novel approach to investigate the stoichiometry of the proteins was mathematically interpreted from the rate of energy transfer between the FRET sensors on their proteins of interest (Ben-Johny et al., 2016). This allowed for the direct assessment of the stoichiometry between exogenous tagged proteins that can be free floating or membrane bound.

The continued investigation will provide insight into the involvement of auxiliary subunits in the intracellular processing of AMPARs. To determine where specific auxiliary subunits are localized in a native environment may establish characteristics between auxiliary subunits. The introduction of the optical technique FRET is a novel approach in assessing the stoichiometry of auxiliary subunits, a question that is not well understood. In Gill et al. (2011) and other previous studies, electrophysiological approaches demonstrated auxiliary subunits association is variable depending on the presence of other auxiliary subunits. Therefore, a future question is to assess auxiliary subunit cooperative and/or competitive binding of AMPAR complexes. This would present another reason for regional distribution of auxiliary subunits and how they coordinate AMPARs and contribute to synaptic plasticity. Auxiliary subunits govern AMPARs in different brain regions and are essential for normal synaptic function. Adding an additional fundamental network of complexity to the central nervous system.

References

Amin JB, Salussolia CL, Chan K, Regan MC, Dai J, Zhou HX, Furukawa H, Bowen ME, Wollmuth LP. Divergent roles of a peripheral transmembrane segment in AMPA and NMDA receptors. J Gen Physiol 149(6):661-680.

Anggono V, Huganir RL. 2012. Regulation of AMPA receptor trafficking and synaptic plasticity. Curr Opin Neurobiol 22(3):461-469.

Ayalon G, Stern-Bach Y. 2001. Functional assembly of the AMPA and kainite receptor is mediated by several discrete protein-protein interactions. Neuron 31(1):103-113.

Ben-Johny M, Yue DN, Yue Dt. 2016. Detecting stoichiometry of macromolecular complexes in live cells using FRET. Nat Commun 7:13709.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA. 2000. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408(6815):936-943.

Defelipe J, Alonso-Nanclares L, Arellano JI. 2002. Microstructure of the neocortex: comparative aspects. J Neurocytol 3-5:299-316.

Gan Q, Dai J, Zhou HX, Wollmuth LP. 2016. The transmembrane domain mediates tetramerization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. J Biol Chem 291(12):6596-6606.

Gan Q, Salussolia CL, Wollmuth LP. 2015. Assembly of AMPA receptors: mechanisms and regulation. J Physiol 593(Pt 1)-39-48.

Gartner A, Cullin L, Lalli G. 2006. Nucleofection of primary neurons. In Balch W, Der C, Hall A. (Eds.), Methods in Enzymology Vol 407: Regulators and Effectors of Small GTPases: Ras Family (pp. 374-388). Cambridge, MA: Academic Press.

Gill MB, Kato AS, Roberts MF, Yu H, Wang H, Tomita S, Bredt DS. 2011. Cornichon-2 modulates AMPA receptor-transmembrane AMPA receptor regulatory protein assembly to dictate gating and pharmacology. J Neurosci 31(18):6928-6938.

Gough NR. 2007. Delivering AMPA receptors to the synapse. Science STKE (415):tw439.

Graham JM. 2002. Fractionation of golgi, endoplasmic reticulum, and plasma membrane from cultured cells in a preformed continuous iodixanol gradient. The ScientificWorld J 2:1435-1439.

Greger IH, Akamine P, Khatri L, Ziff EB. 2006. Developmentally regulated, combinatorial RNA processing modulates AMPA receptor biogenesis. Neuron 51(1):85-97.

Greger IH, Khatri L, Kong X, Ziff EB. 2003. AMPA receptor tetramerization is mediated by Q/R editing. Neuron 40(4):763-764.

Greger IH, Watson JF, Cull-Candy SG. 2017. Structural and functional architecture of AMPA-type glutamate receptors and their auxiliary proteins. Neuron 94:713-730.

Haering SC, Tapken D, Pahl S, Hollmann M. 2014. Auxilairy subunits: shepherding AMPA receptors to the plasma membrane. Membranes (Basal) 4(3):469-490.

Harmel N, Cokic B, Zolles G, Berkefeld H, Mauric V, Fakler B, Stein V, Klocker N. 2012. AMPA receptors commandeer an ancient cargo exporter for use as a auxiliary subunit for signaling. PLoS One 7(1): e30681.

Hatten ME, Gao WQ, Morrison ME, Mason CA. 1998. The cerebellum: purification and coculture of identified cell populations. In Banker G, Goslin K. (Eds.), Culturing Nerve Cells (pp. 419-459) Cambridge, MA: MIT Press.

Henley JM, Wilkinson KA. 2016. Synaptic AMPA receptor composition in development, plasticity and disease. Nat Rev Neurosci 6: 337-350.

Herring BE, Shi Y, Suh YH, Zheng CY, Blankenship SM, Roche KW, Nicoll RA. 2013. Cornichon proteins determine the subunit composition of synaptic AMPA receptors. Neuron 77(6):1083-1096.

Hsieh H, Boehm J, Sato C, Iwatsubo T, Tomita T, Sisodia S, Malinow R. 2006. AMPAR removal underlines Aβ-induced synaptic depression and dendritic spine loss. Neuron 52: 831-843.

Karra D, Dahm R. 2010. Transfection techniques for neuronal cells. J Neurosci 30(18):6171-6177.

Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF. 2011. Mechanism of CaMKII regulation of AMPA receptor gating. Nat Neurosci 14(6):727-735.

Kumar J, Schuck P, Jin R, Mayer ML. 2009. The n-terminal domain of the GluR6-suptype glutamate receptor ion channels. Nat Struct Mol Bio 16(6): 631-638.

Lee HY, Greene LA, Mason CA, Manzini MC. 2009. Isolation and culture of post-natal mouse cerebellar granule neuron progenitor cells and neurons. J Vis Exp (23):990.

Mayer ML. 2011 Emerging models of glutamate receptor ion channel structure and function. Structure 19(10):1370-1380.

McGee TP, Bats C, Farrant M, Cull-Candy SG. 2015. Auxiliary subunit GSG1-L acts to suppress calcium permeable AMPA receptor function. J Neurosci 35(49):16171-16179.

Menuz K, Stroud RM, Nicoll RA, Hay FA. 2007. TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. Science 318(5851):815-817.

Meyerson JR, Chittori S, Merk A, Rao P, Han TH, Serpe M, Mayer ML, Subramaniam S. 2016. Structural basis of kainite subtype glutamate receptor desensitization. Nature 537:576-571.

Moore BS, Mirshahi UL, Ebersole TL, Mirshahi T. 2013. A conserved mechanism for gating in an inotropic glutamate receptor. J Biol Chem 288(26):18842-52.

Nishimura S, Lizuka M, Wakamori M, Akiba I, Imoto K, Barsoumian EL. 2000. Stable expression of human homomeric and heteromeric AMPA receptor subunits in HEK 293 cells. Receptors channels 7(2):139-150.

Paddock SW, Fellers TJ, Davidson MW. 2017. Introductory confocal concepts. MicroscopyU (Nikon Instruments) https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts

Penn AC, Balik A, Wozny C, Cais O, Greger IH. 2012. Activity-mediated AMPA receptor remodeling, driven by alternative splicing in the ligand-binding domain. Neuron 76(3):503-510.

Penn AC, Williams SR, Greger IH. 2008. Gating motions underlie AMPA receptor secretion from the endoplasmic reticulum. EMBO J 27(22):3056-3068.

Pereda AE. 2014. Electrical synapses and their functional interactions with chemical synapses. Nat Rev Neurosci 15(4):250-263.

Preil A, Kollleker A, Ayalon G, Gillor M, Osten P, Stern-Bach Y. 2005. Stargazin reduces desensitization and slows deactivation of the AMPA-type glutamate receptors. J Neurosci 25(10):2682-2686.

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia AS, McNamara JO, Williams SM. 2003. Neuroscience. Sunderland: Sinauer Associates. 832 p.

Ray B, Bailey JA, Sarkar S, Lahiri DK. 2009. Molecular and immunocytochemical characterization of primary neuronal culture from adult rat brain: differential expression of neuronal and glia protein markers. J Neurosci Methods 184(2):294-302.

Reimers JM, Milovanovic M, Wolf ME. 2011. Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. Brain Res 1367:223-233.

Rossman M, Sukumaran M, Penn AC, Veprintsev DB, Babu MM, Greger IH. 2011. Subunit-selective N-terminal domain association organize the formation of AMPA receptor heteromers. EMBO J 30(5): 959-971.

Rouach N, Byrd K, Petralia RS, Elias GM, Adesnik H, Tomita S, Karimzadegan S, Kealey C, Bredt Ds, Nicoll RA. 2016. TARP gamma-8 controls hippocampal AMPA receptor number, distribution and synaptic plasticity. Nat Neurosci 8(11):1525-1533.

Salussolia CL, Gan Q, Kazi R, Singh P, Allopenna J, Furukawa H, Wollmuth LP. 2013. A eukaryotic specific transmembrane segment is required for tetramerization. J Neurosci 33(23):9840-9845.

Salussolia CL, Wollmuth LP. 2012. Flip-flopping to the membrane. Neuron 76:463-465.

Sariyer IK. 2013. Transfection of neuronal cultures. Methods Mol Bio. 1078:133-139.

Schwenk J, Harmel N, Zolles G, Bidl W, Kulik A, Heimrich B, Chisaka O, Jonas P, Schulte U, Fakler B, Klocker N. 2009. Functional proteomics identify cornichon proteins as auxiliary subunits of AMPA receptors. Science 323(5919):1313-1319.

Schwenk J, Baehrens D, Haupt A, Bildl W, Budkkazi S, Roeper J, Fakler B, Schulte U. 2014. Regional diversity and developmental dynamics of the AMPA-receptor proteome in the mammalian brain. Neuron 84:41-54.

Semenov A, Moykkynen T, Coleman SK, Korpi ER, Keinanen K. 2012. Autoactivation of the stargazin-AMPA receptor complex: subunit-dependency and independence from physical dissociation. PLoS One 7(11):e49282.

Serulle Y, Zhang S, Ninan I, Puzzo D, McCarthy M, Khatri L, Arancio O, Ziff EB. 2007. A GluR1-cGKII interaction regulates AMPA receptor trafficking. Neuron 56(4) :607-688.

Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. 2008. Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 14 (8): 837-842.

Shanks NF, Maruo T, Farina AN, Ellisman MH, Nakagawa T. 2010. Contribution of the global subunit structure and stargazin on the maturation of AMPA receptors. J Neurosci 30(7):2728-2740.

Shanks NF, Savas JN, Maruo T, Cais O, Hirao A, Oe S, Ghosh A, Noda Y, Greger IH, Yates III JR, Nakagawa T. 2012. Differences in AMPA and kainate receptor interactomes facilitate identification of AMPA receptor auxiliary subunit GSG1L. Cell Rep 1(6):590-598.

Sobolevsky AI, Rosconi MP, Gouaux E. 2009. X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. Nature 462(7274):745-756.

Soto D, Coombs ID, Renzi M, Zonouzi M, Farrant M, Cull-Candy SG. 2009. Selective regulation of long-form calcium permeable AMPA receptors by an atypical TARP, gamma-5. Nat Neurosci 12(3):277-85.

Tomita S, Chen L, Kawasaki Y, Petralia Rs, Wenthold RJ, Nicoll RA, Bredt DS. 2003. Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. J Cell Biol 161(4):805-16.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. 2010. Glutamate receptor ion channels: structure, regulation, function. Pharmacol Rev 62:405-496.

Twomey EC, Yelshanskaya MV, Grassucci RA, Frank J, Sobolevsky AI. 2016. Elucidation of AMPA receptor-stargazin complexes by cryo-electron microscopy. 353(6294):83-86.

Udea Y, Kwok S, Hayashi Y. 2013. Application of FRET probes in the analysis of neuronal plasticity. Front Neural Circuits 7:162 (pp.1-19)

Vandenberghe W, Nicoll RA, Bredt DS. 2005. Interaction with the unfolded protein response reveals a role for stargazin in biosynthetic AMPA receptor transport. J Neurosci 25(5):1095-1102.

van Spronsen M, Hoogenraad CC. 2010. Synapse Pathology in Psychiatric and Neurologic Disease. Curr Neurol Neurosci Rep 10:207-214.

Vessey JP, Karra D. 2007. More than just synaptic building blocks: scaffolding proteins of the post-synaptic density regulate dendritic patterning. J Neurochem 102(2):324-332.

Wanisch K, Yanez-Munoz RJ. Integration-deficient lentiviral vectors: a slow coming of age. Mol Ther 17(8):1316-1332.

Watson JF, Ho H, Greger IH. 2017. Synaptic transmission and plasticity require AMPA receptor anchoring via its N-terminal domain. elife 6:e23024.

Wittig I, Braun Hp, Schagger H. 2006. Blue native PAGE. Nat Protoc 1(1):418-428.

Yamasaki M, Fukaya M, Yamasaki M, Azechi H, NAtsume R, Abe M, Sakimura K, Watanabe M. 2016. TARP γ-2 and γ-8 differentially control AMPAR density across schaffer collateral/commissural synapses in hippocampal CA1 area. J Neurosci 36(15):4296-4312.

Yan D, Tomita S. 2012. Defined criteria for auxiliary subunits of glutamate receptors. J Physiol 590(1):21-31.

Yang M, Ellenberg J, Bonifacino JS, Weissman AM. 1997. The transmembrane domain of a carbosyl-terminal anchored protein determines localization to the endoplasmic reticiulum. J Biol Chem 272: 1970-1975.

Zeitelhofer M, Karra D, Vessey JP, Jaskic E, Macchi P, Thomas S, Riefler J,
Kiebler M, Dahm R. 2009. High-efficiency transfection of short hairpin RNAs-encoding plasmids into primary hippocampal neurons. J Neurosci Res 87:289–300

Zhang J, Kang DE, Xia W, Okochi M, Mori H, Selkoe DJ, Koo EH. 1998. Subcellular distribution and turnover of presenilins in transfected cells. J Biol Chem 273: 12436-12442.

Zhao Y, Chen S, Yoshioka C, Baconguis I, Gouaux E. 2016. Architecture of fully occupied GluA2 AMPA receptor-TARP complex elucidated by cryo-em. 2016. Nature 536(7614):108-111.

Ziff EB. 2007. TARPs and the AMPA receptor trafficking paradox. Neuron 53(5):627-633.