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Regulation of Glutamate Transporter and Receptor Function by the NHERF Scaffolding Proteins

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Abstract

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Glutamatergic neurotransmission is essential to central nervous system function and dysregulation of glutamatergic signaling is associated with multiple neurodegenerative, pathophysiological, and neuropsychiatric disease states. Levels of glutamate in the brain are controlled by glutamate transporters, and the physiological actions of glutamate are mediated by a variety of ionotropic and metabotropic glutamate receptors. Interestingly, many of these transporters and receptors possess motifs indicative of potential binding to PDZ domains, suggesting that PDZ scaffold proteins might be critical regulators of glutamate transporter and receptor function. In the studies described in this dissertation, my colleagues and I identified and characterized novel PDZ-interacting partners for the astrocytic glutamate transporter GLAST and the Group II metabotropic glutamate receptors, mGluR2 and mGluR3. Specifically, proteomic screens identified the Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF-1 and NHERF-2) as candidate interacting partners for GLAST and Group II mGluRs. Subsequent studies on cultured rat cortical astrocytes revealed that NHERF-2 is abundantly expressed in these cells and enhances GLAST stability and function. Moreover, interactions between Group II mGluRs and NHERF proteins were validated in a cellular context, and disruption of the C-terminal PDZ-interacting motif of mGluR2 was found to enhance receptor-mediated activation of AKT in astrocytes; however, it was unclear if this enhancement was due to disruption of associations with the NHERF proteins, since the effects of the mutation were not recapitulated by knock-out or knockdown of NHERF-1 or NHERF-2. We furthermore examined how the NHERF proteins might regulate Group II mGluRs *in vivo*. An electron microscopic examination of the cellular and sub-cellular distribution of Group II mGluRs in the mouse cortex in wild-type (WT), NHERF-1 KO, and NHERF-2 KO mice, revealed that loss of NHERF-2 led to a modest redistribution in the cellular targeting of Group II mGluRs without altering overall Group II mGluR expression. These studies shed light on the physiological significance of scaffold protein interactions with glutamate transporters and receptors, and thereby enhance our understanding of the molecular mechanisms underlying both normal regulation and potential dysregulation of glutamatergic neurotransmission.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. Glutamate and the CNS

1.1.A. Glutamatergic neurotransmission

Glutamate is the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS) and mediates the majority of chemical neurotransmission at excitatory synapses in the brain. However, given that glutamate is also an amino acid found common throughout the body as well as a by-product of various metabolic cascades, it took many years of research to conclusively demonstrate that glutamate serves as a classical neurotransmitter. The criteria for a neurotransmitter is as follows: 1) it is synthesized within and released from the presynaptic neuron, 2) the neurotransmitter can be isolated from the nerve terminal and pharmacologically characterized 3) the neurotransmitter must act on the postsynaptic neuron to induce a specific set of events that mimic those that transpire following direct stimulation of the presynaptic neuron 4) a competitive antagonist can block the actions of the neurotransmitter in a dose dependent fashion and 5) a specific clearance mechanism exists to remove it from the synapse (Squire L.R. et al., 2002).

In fulfillment of these criteria, glutamate was recognized as a neurotransmitter by the 1970s (Meldrum, 2000). Its biosynthesis can result from the conversion of glucose into glutamate in the presynaptic neuron, as well as its conversion from glutamine to glutamate by a glutaminase enzyme that is found in presynaptic neurons (Daikhin and Yudkoff, 2000), thereby fulfilling criteria 1. Early studies in 1959 revealed that application of glutamate via iontophoresis onto spinal neurons elicited spike discharges from those neurons that were similar to the spiking pattern observed following direct electrical stimulation of the neurons (Curtis et al., 1959) (criteria 3). However, it was not until the 1960s that more direct evidence that glutamate was a neurotransmitter had emerged, whereby Curtis and Watkins demonstrated that certain glutamate analogues, such as NMDA, could also elicit spike discharges (Curtis and Watkins, 1963; Fonnum, 1984; Krnjevic and Phillis, 1963).

1.1.B. CNS Sources of Glutamate

Although glutamate is the most abundant excitatory neurotransmitter in the mammalian CNS, it does not readily cross the blood-brain-barrier and is therefore generally synthesized within the CNS (Hawkins, 2009; Smith et al., 1987). The carbon back-bone for glutamate is derived from glucose stores, in which glucose can be converted into acetyl-coA and shuttled into the tricarboxylic acid (TCA) cycle to generate α-ketoglutarate, which is subsequently converted into glutamate via glutamate dehydrogenase (Daikhin and Yudkoff, 2000) (see Figure 1.1). In astrocytes, glycogen and lactate also act as a source of glucose or pyruvate, which in turn can ultimately feed into the TCA cycle to generate glutamate. In contrast, neurons do not have glycogen and instead use lactate as an alternative entry point into glycolysis and the TCA cycle (Figure 1.1). Another key source of glutamate in the brain, and in particular in neurons, is from glutamine, which can be converted into glutamate by phosphate-activated glutaminase (PAG) (Daikhin and Yudkoff, 2000) (see Figure 1.1). Astrocytes will often convert glutamate into glutamine and then shuttle the glutamine to the neurons for replenishment of glutamate, as well as metabolic support (see section 1.2 for more detail). In sum, these metabolic pathways act to generate glutamate from energy sources and convert it into forms that are not potentially damaging to neurons.

1.1.C. Regulation of Extracellular Glutamate Concentrations

The determination of extracellular glutamate concentration depends in large part on the compartment in which it is being measured and the method used to determine the concentration (Moussawi et al., 2011). Following presynaptic depolarization and release of glutamate, synaptic glutamate levels may rapidly rise to as much as 1 mM (McKenna, 2007) and then quickly decline (~10 ms period) to approximately 25 nM levels (Herman et al., 2011; Moussawi et al., 2011). However, as recently reviewed by the Kalivas group, basal extracellular glutamate concentrations

outside of the synaptic cleft can range between 0.2 and 30 μM, in which this broad range can most readily be explained by different measurement techniques and/or the biological compartment and its complement of glutamate transporters, antiporters, receptors and the area of the extracellular space that is being sampled (Moussawi et al., 2011). This appears to be the more current consensus in the field as a concurrent report reached a similar conclusion (Herman et al., 2011).

Of note, basal extracellular glutamate levels are maintained independent of vesicular glutamate release, as evidenced by studies in which glial glutamate transporters, voltage-gated calcium and voltage-gated sodium channels, as well as vesicular glutamate transporters were blocked and extracellular glutamate levels still increased (Baker et al., 2002; Jabaudon et al., 1999). It is now thought that the cystine-glutamate exchanger ($-X_C$) is the antiporter that is primarily responsible for maintaining basal extracellular glutamate levels. The $-X_C$ is an anionic amino acid antiporter that exchanges one extracellular cystine for one intracellular glutamate and thus acts as a source of non-vesicular released glutamate (Bannai, 1986; Sato et al., 1999). Unlike the sodium dependent glutamate transporters (EAAT1-EAAT5, see section 1.4), the $-X_C$ is sodium independent and electrogenically neutral. The $-X_C$ is comprised of two subunits, xCT and 4F2hc (Sato et al., 1999) and has been shown to be widely distributed throughout the mouse and human brain, where it is found in both neurons and glia (Burdo et al., 2006). Once cystine is inside the cell, it is then reduced to cysteine, which serves as the rate-limiting step in glutathione synthesis, along with glutamate and glycine (Meister, 1995). Interestingly, glutathione is considered to be the primary endogenous antioxidant in the brain and is instrumental in regulating redox states of cells (Dringen, 2000). Therefore, the driving force behind $-X_C$ dependent exchange of glutamate can be the redox state of the cells.

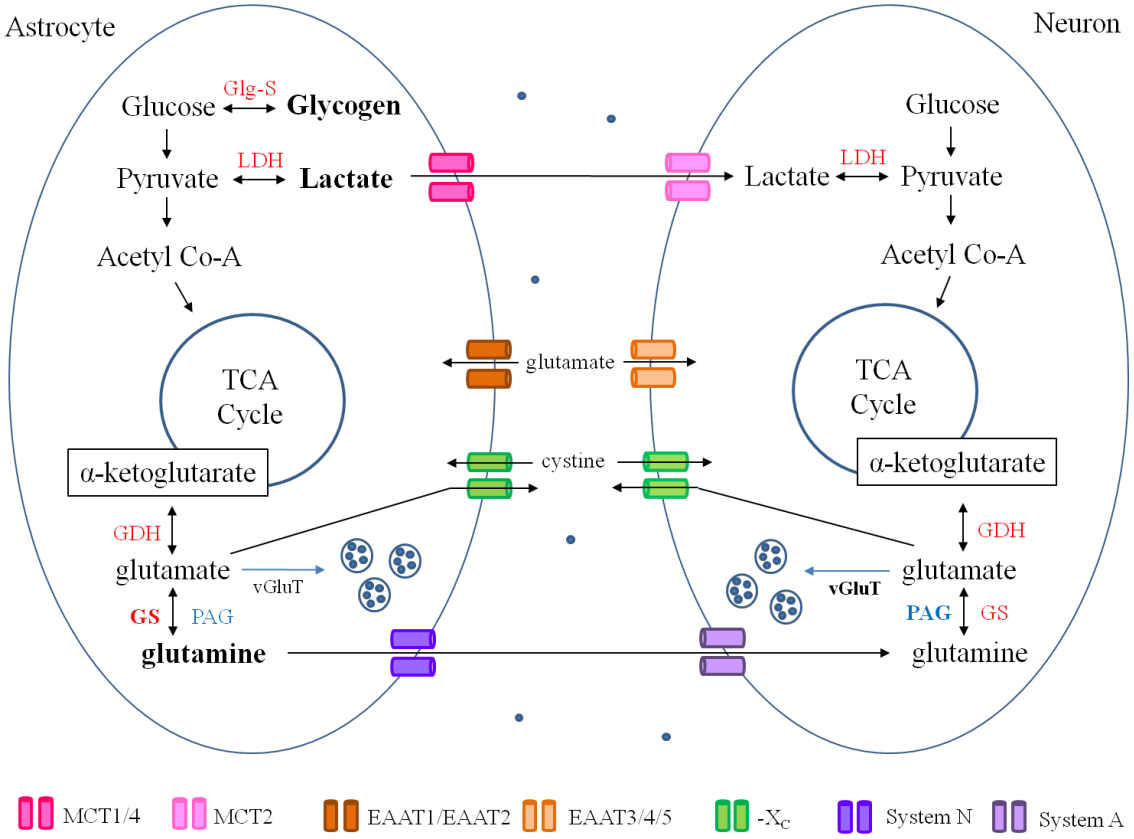

Figure 1.1. Overview of glutamate biosynthesis, storage, and release in the CNS. A hypothetical astrocyte and neuron are shown. The carbon backbone of glutamate is generated from glucose via its catabolism in the tricarboxylic acid (TCA) cycle in both astrocytes and neurons via the reciprocal conversion of α-ketoglutarate into glutamate by glutamate dehydrogenase (GDH). Glucose and pyruvate, an intermediate of glycolysis, can be derived from glycogen and lactate stores in astrocytes, whereas in neurons lactate is the only alternative energy source for glycolysis. Glycogen and glucose are interconverted via the enzyme glycogen synthase (Glg-S), while lactate and pyruvate are interconverted via the enzyme lactate dehydrogenase (LDH). Lactate is also shuttled from astrocytes to neurons via the MCT family transporters. Once generated, glutamate can be converted into glutamine, via the enzyme glutamate synthetase (GS), where it is then preferentially shuttled from astrocytes to neurons via the System N transporter system in astrocytes and the System A transporter in neurons.

Phosphate-activated glutaminase (PAG) enzymes will then covert the glutamine into glutamate, thereby replenishing neuronal glutamate levels, without inducing excitotoxicity. In excitatory neurons, glutamate may also be packaged into vesicles by the vescicular glutamate transporters (vGluT); astrocytes have also been reported to package in glutamate into vesicles, although this is more controversial and not widely accepted. See section 1.2 on astrocytes for more detail. Rather than synthesizing glutamate, both astrocytes and neurons can also uptake extracellular glutamate via the excitatory amino acid transporters, EAAT1-5. Finally, both astrocytes and neurons express the cystine-glutmate exchanger $-X_C$, which maintains basal extracellular glutamate levels by exchanging one glutamate for an intracellular cystine. Bolded metabolites or enzymes correspond to the cell type that is preferentially enriched in the substance.

1.2. Astroctyes

1.2.A. Overview and Function

The function of astrocytes in the central nervous system has greatly expanded from the original view that astrocytes, as a type of glia, were merely the “glue” that holds neurons together. It is now widely appreciated that astrocytes not only provide integral metabolic and trophic support, but also induce the development of the blood-brain-barrier and are key players in sculpting synaptic neurotransmission, with an active and lively debate concerning whether they can secrete/exocytose "gliotransmitters" that regulate neuronal communication.

The view that astrocytes provide key metabolic support for neurons is expanding to also link astrocytic-mediated metabolic support with neuronal activity. The fact that 20% of cardiac output goes to the human brain, which represents only 2% of body mass, clearly highlights the energy burden that brain cells require (Quaegebeur et al., 2011). Astrocytes, but not neurons, can store glycogen that acts a readily-available pool of energy, which can be rapidly catabolized into lactate and/or pyruvate (Vilchez et al., 2007) (see Figure 1.1). In times of high metabolic requirements and/or pathological glucose deprivation (for example ischemia or stroke), astrocytes will secrete lactate and/or pyruvate, which is then shuttled to neurons for their use (Brown et al., 2004a; Izumi et al., 1997; Magistretti and Pellerin, 1999). The importance of astrocytic lactate production and its extracellular release has been highlighted in elegant studies demonstrating the requirement of astrocytic-mediated lactate release in long term memory formation (Suzuki et al., 2011). Glutamate uptake by astrocytes can also induce astrocytic lactate release, which has been hypothesized to link synaptic activity (i.e. uptake of synaptic glutamate) with astrocytic lactate release and metabolic support of neurons (Magistretti, 2009; Magistretti and Pellerin, 1999). More recently, a soluble adenylyl cyclase that is sensitive to bicarbonate levels, and therefore intracellular pH, has been shown to increase intracellular cAMP levels and trigger glycogen breakdown and lactate efflux (Choi et al., 2012). As high metabolic activity is often associated

with dramatic changes in acid/base homeostasis, this provides an additional mechanism, outside of glutamate uptake, coupling neuronal activity with astrocytic metabolic support. Additionally, astrocytes can convert glutamate into glutamine, providing an important mechanism for neurotransmitter replenishment in neurons, as well as clearance of synaptic and extrasynaptic glutamate. Glial glutamate transporters uptake glutamate (see section 1.4), which is subsequently converted by astrocytic glutamine synthetase into glutamine. Glutamine is then transported out of astrocytes via the System N Transporter 1, and taken up by neighboring neurons via System A Transporter 1 and 2 (Reimer et al., 2000). Neurons can then metabolize the glutamine into more glutamate or GABA (Chaudhry et al., 2002), thereby replenishing neurotransmitter stores (see Figure 1.1).

Astrocytes have long been known to be provide trophic support to neurons (Banker, 1980; Muller et al., 1995) as well as other CNS-specific cell types including oligodendrocyte precursor cells (Arai and Lo, 2010); however, the mediating trophic factors are still being identified (Barres, 2008). As reviewed by Muller and colleagues, astrocytes have been shown to secrete many chemicals that have been identified as growth factors including nerve growth factor (NGF), basic fibroblast-like growth factor (bFGF), ciliary neurotrophic factor (CNTF, relatively specific for astrocytes in spinal cord and optic nerve), insulin-like growth factor (IGF), and transforming growth factor β (TGF-β) (Muller et al., 1995). Astrocytes can also secrete certain growth factors that have differential selectivity for various neuronal populations. For example, secretion of mesencephalic astrocyte-dervided neurotrophic factor can selectively protect dopaminergic neurons in the substania nigra (MANF) (Petrova et al., 2003). Finally, it has been shown that selective mutation of the epidermal growth factor (EGF) receptor from forebrain but not midbrain astrocytes leads to increased astrocyte apoptosis, ultimately inducing a neurodegenerative phenotype (Wagner et al., 2006). This study not only highlights the “trophic”

role that astrocytes can play *in vivo*, but also speaks to the functional heterogeneity of astrocytes *in vivo*.

Astrocytes are the primary liaison between the peripheral environment (blood) and the brain and can also induce the formation of the blood brain barrier (BBB), which serves as the cellular interface between the brain and the periphery (Abbott, 2002; Nico and Ribatti, 2012). The BBB is comprised of endothelial cells found within the vasculature of the CNS that are connected via tight junctions, thereby creating a nearly impermeable barrier that effectively restricts most paracellular transport and only permits transcellular transport (Abbott, 2002). A large complement of specialized transporters are present on endothelial cells to facilitate the transport of small polar solutes such as glucose, or neutral amino acids, as well as the low density lipoprotein receptor (LDLR) and transferrin receptors to name a few (Nico and Ribatti, 2012). At the interface of this transport system, astrocytes have small end-feet processes that envelop the endothelial cells, allowing for the uptake of these selective substrates from the blood and the passage of selective substrates from astrocytes (Nico and Ribatti, 2012). Importantly, astrocytes help to induce the creation of the BBB and thus are integral to its formation. For example, recently it was shown that perivascular astrocytes can release sonic hedgehog, which in turn binds to and activates sonic hedgehog receptors on endothelial cells to promote BBB formation via the activation of the transcription factor Gli-1 and the subsequent upregulation of tight junctional proteins (Alvarez et al., 2011).

Perhaps even more challenging of the notion that astrocytes play a merely "supportive" in the CNS is the ongoing debate as to what extent can astrocytes actively regulate neuronal synaptic activity. A significant amount of work from the Barres laboratory has endeavored to tackle this question. Beginning with the observation that the formation of functional, yet silent synapses, requires the co-culture of astrocytes with retinal ganglion cells (Pfrieger and Barres, 1997), the Barres research group went on to discover that the astrocytic release of

thromobospondins is sufficient to induce the formation of these silent synapses (Christopherson et al., 2005). More recently, the Barres team has continued this work by demonstrating that the release of certain glypicans from astrocytes can induce ionotropic glutamate AMPA receptor subunit upregulation and insertion into the plasma membrane, effectively transitioning the silent synapses into functional ones (Allen et al., 2012). Together, these data support the ability of astrocytes to promote synaptogenesis and thereby regulate neuronal connectivity and activity.

In further support of their role as integral players in synaptic neurotransmission, many studies have examined the ability of astrocytes to release gliotransmitters (Araque et al., 1999; Barres, 2008; Hamilton and Attwell, 2010), although it has been exceptionally controversial (Parpura and Zorec, 2010). Gliotransmitters that have been shown to be released from astrocytes include D-serine, glutamate, ATP and its metabolite, adenosine, as well as some peptides such as brain-derived neurotrophic factor (BDNF) (Barres, 2008; Hamilton and Attwell, 2010; Parpura and Zorec, 2010). Release of these gliotransmitters has been shown to be meaningful in the context of neuronal function, as astrocytic release of D-serine in the CA1 region of the hippocampus has been shown to be necessary for NMDA-mediated LTP in surrounding neurons, since D-serine is a necessary co-factor for NMDA activation (Henneberger et al., 2010). Astrocytic adenosine can modulate sleep homeostasis (Halassa et al., 2009).

Although it is clear that astrocytes can secrete many substances, it has been quite controversial as to whether these substances are exocytosed in a mechanism analogous to neurotransmitter release from pre-synaptic nerve terminals. A significant body of evidence supports that elevations in intracellular calcium are required for gliotransmitter release, as determined using a variety of approaches that block intracellular calcium signaling such as chelation of calcium with BAPTA (Hamilton and Attwell, 2010; Parpura and Zorec, 2010). However, it is less understood whether astrocytes can truly exocytose gliotransmitters in a vesicular-mediated fashion (Hamilton and Attwell, 2010). In support of this notion, evidence is

accumulating that astrocytes express the machinery required for the packaging of glutamate and/or other gliotransmitters into vesicles, as well as the machinery necessary for its release, analogous to release of neurotransmitters from pre-synaptic neurons (Hamilton and Attwell, 2010). For example, it has recently been shown through immunogold labeling of astrocyte processes that they can contain synaptic-like microvesicles which are immunopositive for vGluT1 (Ormel et al., 2012). Additionally, astrocytes have been found to express machinery for calcium-mediated exocytosis including components of SNARE complexes such as synaptobrevin 2, syntaxin, and SNAP-23 (Montana et al., 2006).

However, alternative routes of transmitter release have been proposed, including reversal of glutamate transporters, albeit under pathophysiological conditions such as ischemia (Rossi et al., 2000) , efflux through volume-regulated anion channels, ionotropic purinergic $P2X_7$ receptors, or through gap junctional hemichannels (although this does not appear to require elevations in intracellular calcium), as well as lysosomal exocytosis, as reviewed by (Hamilton and Attwell, 2010). For example, it was recently shown that activation of protease activated 1 receptors (PAR) on cultured hippocampal astrocytes can induce the release of astrocytic glutamate that is independent of vesicular-mediated endocytosis (Oh et al., 2012). In summary, it is still quite contentious as to whether astrocytes can exocytose gliotransmitters and how this may be meaningful for neuronal function *in vivo*.

1.2.B. Tripartite Synapse

Given the emerging evidence that astrocytes can participate in bi-directional communication between neurons and themselves, and that astrocytes are intimately associated with synapses from an anatomical perspective, has led to the identification of a specialized compartment in the central nervous system, in which astrocytes physically associate with the pre- and post-synaptic neuronal elements, termed the tripartite synapse (Araque et al., 1999; Perea et

al., 2009). The tri-partite synapse refers to the ability of astrocytes to both respond to, integrate information from, and regulate both the pre- and post-synaptic neuronal elements, ultimately arguing that brain function can be explained by the coordinated activity of neurons and glia (Perea et al., 2009). As discussed above, it is still controversial as to what extent astrocytes are active participants in sculpting and shaping synaptic neurotransmission, but increasing evidence supports the notion that astrocytes can regulate synaptic neurotransmission and plasticity and are thus integral to coding information in central nervous system, alongside of neurons.

1.2.C. Perisynaptic Astrocyte Processes

Astrocytic endfeet that ensheath pre-synaptic and post-synaptic neuronal elements represent a particular astrocyte compartment termed the perisynaptic astrocyte process or PAP. This compartment has been shown to be highly dynamic in *in vitro* studies (Lavialle et al., 2011). The molecular composition of the PAP is receiving increasing interest, as certain transporters, receptors, cytosolic proteins, and enzymes have been shown to be localized to this compartment. For example, immunoelectron microscopy studies have revealed that glutamate transporters, including EAAT1/GLAST and EAAT2/GLT-1 (Chaudhry et al., 1995; Cholet et al., 2002; Lavialle et al., 2011), as well as metabotropic glutamate receptors mGluR3 and mGluR5 (Lavialle et al., 2011), are expressed in these compartments in rodent hippocampus. Actin is the primary cytoarchitectural element in this compartment, whose remodeling is thought to underlie astrocyte endfeet process motility dynamics (Derouiche and Frotscher, 2001). Moreover, the actin-binding protein ezrin has been found to be especially enriched in this astrocytic compartment (Derouiche et al., 2002; Derouiche and Frotscher, 2001). A number of proteins involved in metabolism are also localized in the PAP. In hippocampal slices, GLT-1 has also been shown to co-localize in PAPs with various glycolytic enzymes, mitochondria, and the sodium potassium ATPase (Na+/K+ ATPase), implicating the coupling of glutamate transport with metabolic activity (Genda et al.,

2011). Likewise, glutamine synthetase is also expressed in the PAP compartment, providing additional support for links between the uptake of glutamate and its metabolism (Derouiche and Frotscher, 1991). Interestingly, the extent of glial coverage of synapses is thought to vary within (Ventura and Harris, 1999) and across brain regions, thus highlighting that the anatomy of the PAPs themselves may vary.

1.3. Excitotoxicity

When excess glutamate is present in the extracellular environment, neurons may die via a distinct type of cellular death known as excitotoxicity. Evidence that glutamate could lead to neuronal cell death first emerged with studies of the application of monosodium glutamate (MSG) to the retina, where it was found by Lucas and Newhouse that the inner neuronal layer of the retina was destroyed (Lucas and Newhouse, 1957). However, the notion that monosodium glutamate could destroy neurons in the cerebrum emerged in 1969 when James Olney exposed infant macaques to subcutaneous administration of MSG, which lead to the ablation of the hypothalamus, a region early on in development that is not well-protected by the blood-brain-barrier (Olney and Sharpe, 1969). Although many papers state that the term "excitotoxicity" was coined by James Olney in his 1969 study (Sattler and Tymianski, 2001), this term was actually not ever used, although the study did serve as the first evidence that glutamate could selectively kill neurons in the brain. The term excitotoxicity was later popularized by both Olney (Olney, 1986) and Dennis Choi (Choi, 1992) and is now commonly used. It is widely believed that excitotoxicity contributes to the underlying pathophysiology of many neurological conditions, including epilepsy, stroke (hypoxia or ischemia), and chronic neurodegenerative conditions.

The underlying mechanism of excitotoxicity was not fully appreciated until the glutamate receptors were identified. Prolonged stimulation of ionotropic NMDA receptors (see section 1.5) can lead to the influx of excessive sodium and calcium, which in turn can activate calcium-

sensitive enzymes. However, all ionotropic glutamate receptors, including the AMPA and kainite classes in addition to the NMDA class, can mediate neurotoxicity, primarily due to the influx of calcium (Sattler and Tymianski, 2001). The molecular and cellular mechanisms underlying excitoxicity include osmotic swelling due to alterations in intracellular calcium and sodium concentrations, oxidative stress, mitochondrial dysfunction and caspase activation (Arundine and Tymianski, 2003; Dong et al., 2009).

1.4. Glutamate Transporters

1.4.A. Overview

Glutamate is cleared from extracellular regions via a combination of diffusion and re-uptake mechanisms (Watkins and Evans, 1981). In the early 1990s, the glutamate transporters were identified and cloned (Arriza et al., 1997; Fairman et al., 1995; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). Currently, five glutamate transporters have been identified as the principle regulators of extracellular glutamate levels and have been so named excitatory amino acid transporters EAAT-1 (also known as GLAST), EAAT-2 (also known as GLT-1), EAAT-3 (also known as EAAC-1), EAAT-4, and EAAT-5.

Glutamate transporters are members of the secondary active transport family and use energy stored within electrochemical gradients to actively transport one glutamate, three sodium ions and one proton into the cell in exchange for the removal of one potassium ion per transport cycle (Levy et al., 1998; Zerangue and Kavanaugh, 1996). This generates an electrogenic current which can be measured via recording of an inward sodium current or intracellular acidification (Zerangue and Kavanaugh, 1996). EAATs also have a substrate-activated chloride current that is separate from the current generated during the transport of glutamate (Jiang and Amara, 2011), thereby providing another means to measure glutamate transporter activity.

Genetic studies have shed light on the functions of the individual transporter sub-types, revealing a predominant role for EAAT1/GLAST and EAAT2/GLT-1 in controlling synaptic glutamate levels. In particular, mice lacking either GLAST or GLT-1 show distinctive patterns of neurodegeneration and are much more susceptible to seizures and excitotoxic cellular death than wild-type mice (Rothstein et al., 1996; Tanaka et al., 1997). Moreover, individual knockouts of the other glutamate transporters, EAAT3-5, result in significantly milder seizure-prone phenotypes and the absence of any obvious signs of neurodegeneration (Peghini et al., 1997; Takayasu et al., 2005), ultimately speaking to the importance of the astrocytic glutamate transporters in clearing extracellular glutamate. Dysregulation of glutamate transporter function has been linked to many neurological and neurodegenerative disease states, including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease, Parkinson's Disease, stroke and ischemia, schizophrenia and epilepsy (Beart and O'Shea, 2007; Maragakis and Rothstein, 2001; Sheldon and Robinson, 2007).

1.4.B. Distribution

The tissue expression patterns of the glutamate transporters are variable, with GLAST/EAAT1, GLT-1/EAAT2, and EAAC1/EAAT3 being the predominant glutamate transporters found in the cerebral cortex, albeit at various stages of development. In rodent models, it was found that GLAST is moderately expressed throughout the brain in early development, whereas its expression is later primarily restricted to the cerebellum in adults (Furuta et al., 1997b; Lehre and Danbolt, 1998). In contrast, GLT-1 is expressed at low levels in the developing brain, yet is upregulated to become the predominant glial transporter subtype in the adult rodent brain (Danbolt et al., 1992; Lehre and Danbolt, 1998). Similarly, in the developing human brain, EAAT1 is more diffusely distributed throughout the cerebrum; however, it is the only glutamate transporter expressed in the periventricular region (Bar-Peled et al., 1997).

Relative to EAAT1, EAAT2 is more highly expressed throughout the developing cerebrum and cerebellum (Bar-Peled et al., 1997). EAAC1 has been detected in the rodent forebrain, diencephalon, and hindbrain during prenatal and postnatal development, but its overall expression was found to be higher in newborn postnatal rats, relative to adults (Furuta et al., 1997b). An analogous expression pattern has been observed for EAAT3 in the developing cortex, basal ganglia, and hippocampus (Bar-Peled et al., 1997). In the adult rodent, EAAT4 is expressed almost exclusively in the cerebellum (Fairman et al., 1995) and its levels have been shown to increase with age (Furuta et al., 1997b). Some EAAT4 immunoreactivity has been observed in the human developing cortex, hippocampus, and cerebellar cortex (Bar-Peled et al., 1997). Interestingly, out of the four glutamate transporters, expression of EAAT5 is restricted to the retina, where it is found within photoreceptors and bipolar cells (Arriza et al., 1997; Pow and Barnett, 2000).

At the cellular and subcellular level, EAAT1/GLAST and EAAT2/GLT-1 are generally expressed in astrocytes (Chaudhry et al., 1995; Danbolt et al., 1992; Lehre et al., 1995) whereas EAAT-3-5 exhibit their primary expression in neurons (Arriza et al., 1997; Rothstein et al., 1994; Yamada et al., 1996). Initial characterizations of GLAST and GLT-1 localization in the adult rat revealed that GLT-1 was significantly more expressed in astrocytes in the hippocampus, relative to Bergman glia in the cerebellum (Chaudhry et al., 1995). Post-natal expression of GLAST immunoreactivity has been observed in Bergmann glia of the cerebellum (Furuta et al., 1997b), as well as in some glia in the forebrain. In the adult cerebrum, GLT-1 localizes exclusively to glia, whereas it has some distribution in unmyelinated axons in early prenatal development (Furuta et al., 1997b). More recently, an elegant study using transgenic reporter mice for GLAST and GLT-1 revealed that GLAST and GLT-1 do not substantially overlap in adult rodent astrocytes and that GLAST promoter activity also persists in radial glial cells and oligodendrocytes near white matter tracts in the adult rodent forebrain (Regan et al., 2007). Both EAAT3 and EAAT4 tend to be

localized on post-synaptic elements, with preferential somatodendritic localizations (Furuta et al., 1997a), although some EAAT4 immunoreactivity has also been detected in axons and boutons in Purkinje cells in the cerebellum (Furuta et al., 1997a; Furuta et al., 1997b). In contrast, EAAT5 tends to be distributed on pre-synaptic elements, as it is found on cell bodies, pre-terminal axons, and axon terminals of bipolar cells in the retina (Pow and Barnett, 2000).

1.5. Ionotropic Glutamate Receptors

1.5.A. Overview

The first class of glutamate receptors to be identified was the ionotropic glutamate receptor (iGluR) family, which are ligand-gated cation channels that can mediate fast responses to glutamate, relative to metabotropic glutamate receptors (see section 1.6). In 1989, the first ionotropic glutamate receptor subunit was cloned (Hollmann et al., 1989) whereby many additional glutamate receptors subunits were subsequently cloned and identified (Hollmann and Heinemann, 1994). There are four classes of ionotropic glutamate receptors found in the brain, which are grouped based upon pharmacological selectivity and structural homology including the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (Hollmann and Heinemann, 1994), kainate (KAR), *N*-methyl-D-aspartate (NMDA) receptors, and the δ receptors. NMDA receptors are thought to act as coincidence detectors and are therefore implicated in synaptic plasticity (Scannevin and Huganir, 2000). AMPA and kainate receptors mediate rapid, fast-acting responses to glutamate, while NMDA receptors mediate a slower-acting response to glutamate. Less is known about the δ delta receptors as their overall function is not well understood. Intriguingly, δ delta receptors appear to be important in cerebellar function, independent of their properties as an ion channel and/or the ligand binding domain. For a review of ionotropic glutamate receptors, see here (Dingledine et al., 1999; Traynelis et al., 2010).

1.5.B. Distribution

There are many subunits for the four various iGluR families and most of these subunits are fairly widely distributed throughout the brain. In rodents, two subunits of the NMDA receptor, the GluN1 (NR1) and the GluN2A/B (NR2A/B), have been shown to be widespread throughout the central nervous system (Petralia et al., 1994a; Petralia et al., 1994b) including olfactory bulb, cerebral cortex, hippocampus, caudate-putamen, thalamus (GluN1), hypothalamus (GluN1), colliculi (GluN1), and many brainstem nuclei, although interestingly, the cerebellum has been shown to be devoid of GluN2A/B staining. Like-wise the distribution of some of the subunits of the AMPA receptor, GluA1 (GluR1), GluA2/3 (GluR2/3), and GluA4 (GluR4) in the rodent central nervous system is also fairly wide-spread (Petralia and Wenthold, 1992) with predominant staining observed in the forebrain, as well as some staining in the cerebellum. Some expression pattern differences have been observed such as the presence of GluA1 and GluA4 in Bergmann glia in the cerebellum, while GluA2/3 labeling has been primarily observed in Purkinje neurons and not glia (Petralia and Wenthold, 1992).

Ionotropic glutamate receptors tend to be localized to post-synaptic elements, including spines and dendrites, where they are positioned to mediate fast-acting responses to synaptically released glutamate. A significant volume of work has demonstrated that many of the iGluRs are selectively localized to the post-synaptic density (PSD) which is a dense network of scaffolding proteins, cytoskeletal elements, and receptors (Scannevin and Huganir, 2000; Sheng, 2001; Sheng and Pak, 1999). Of all the iGluR classes, NMDA receptors tend to be enriched at the PSD, whereas AMPA receptors tend to be localized to the PSD and perisynaptic compartments (Petralia and Wenthold, 1992). Moreover, AMPA receptors are mobile within the PSD compartment, as in the case during long term potentiation, when certain AMPA receptors can be redistributed from the intracellular compartment to the plasma membrane to facilitate strengthening of synapses, or

in the case of long term depression, AMPA receptors can be removed from the plasma membrane via endocytosis (Malinow and Malenka, 2002).

More recently, the characterization of iGluR localization in presynaptic terminals has been reviewed (Pinheiro and Mulle, 2008). For example, KARs have also been shown to be localized on presynaptic GABAergic terminals in the rat globus pallidus (Jin and Smith, 2007), where they can function as heteroreceptors. KARs have also been found in presynaptic terminals of mossy fiber neurons that synapse with neurons in CA3 in the hippocampus (Pinheiro and Mulle, 2008).

1.6. Overview of Classical G Protein-Coupled Receptor (GPCR) Signaling Pathways[1]

In the classical view of G protein-coupled receptor (GPCR) signaling, an agonist binds to extracellular and/or transmembrane regions of the receptor, leading to its interaction with heterotrimeric G proteins. The GPCR acts as a guanine nucleotide exchange factor, catalysing the exchange of GDP for GTP on the Gα subunit and inducing dissociation of the Gα and Gβγ subunits from each other and from the GPCR (Figure 1.3A). Activated α-GTP subunits, of which there are multiple subtypes, including $G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$ and $G\alpha_q$, subsequently bind to and regulate the activity of effectors such as adenylyl cyclase, RhoGEF and phospholipase Cβ (PLCβ). These modulate downstream effectors directly or by generating second messengers (such as cyclic AMP, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) that modulate further downstream effectors, such as protein kinase A (PKA) and protein kinase C (PKC). Following their liberation from the heterotrimeric G protein complex, the βγ subunits can also bind to and regulate certain downstream effectors, such as ion channels and PLCβ.

[1] This section has been reproduced with permission from **Ritter SL**, Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10:** 819-830. Minor modifications have been made.

G protein-mediated signaling by agonist-activated GPCRs can be terminated through GPCR phosphorylation by GPCR kinases (GRKs) and concomitant GPCR association with arrestins, which interact with clathrin and the clathrin adaptor AP2 to drive GPCR internalization into endosomes (Hanyaloglu and von Zastrow, 2008; Moore et al., 2007; Premont and Gainetdinov, 2007; Reiter and Lefkowitz, 2006) (see Figure 1.3B). GPCR internalization regulates the functional process of receptor desensitization. Recruitment of arrestins to activated GPCRs can also lead to the initiation of distinct arrestin-mediated signaling pathways, including activation of the mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) pathway. Following internalization after association with arrestins, GPCRs can be trafficked to lysosomes, where they are ultimately degraded, or to recycling endosomes for recycling back to the cell surface in the functional process of resensitization — whereby the cell is resensitized for another round of signaling. Interestingly, 'biased' agonists have been recently characterized that specifically activate G protein-mediated signaling pathways over arrestin-mediated GPCR signaling pathways, or vice versa (Urban et al., 2007; Violin and Lefkowitz, 2007). This new concept illustrates the importance of characterizing all GPCR downstream signaling pathways in order to fully exploit the therapeutic potential of clinically important receptors.

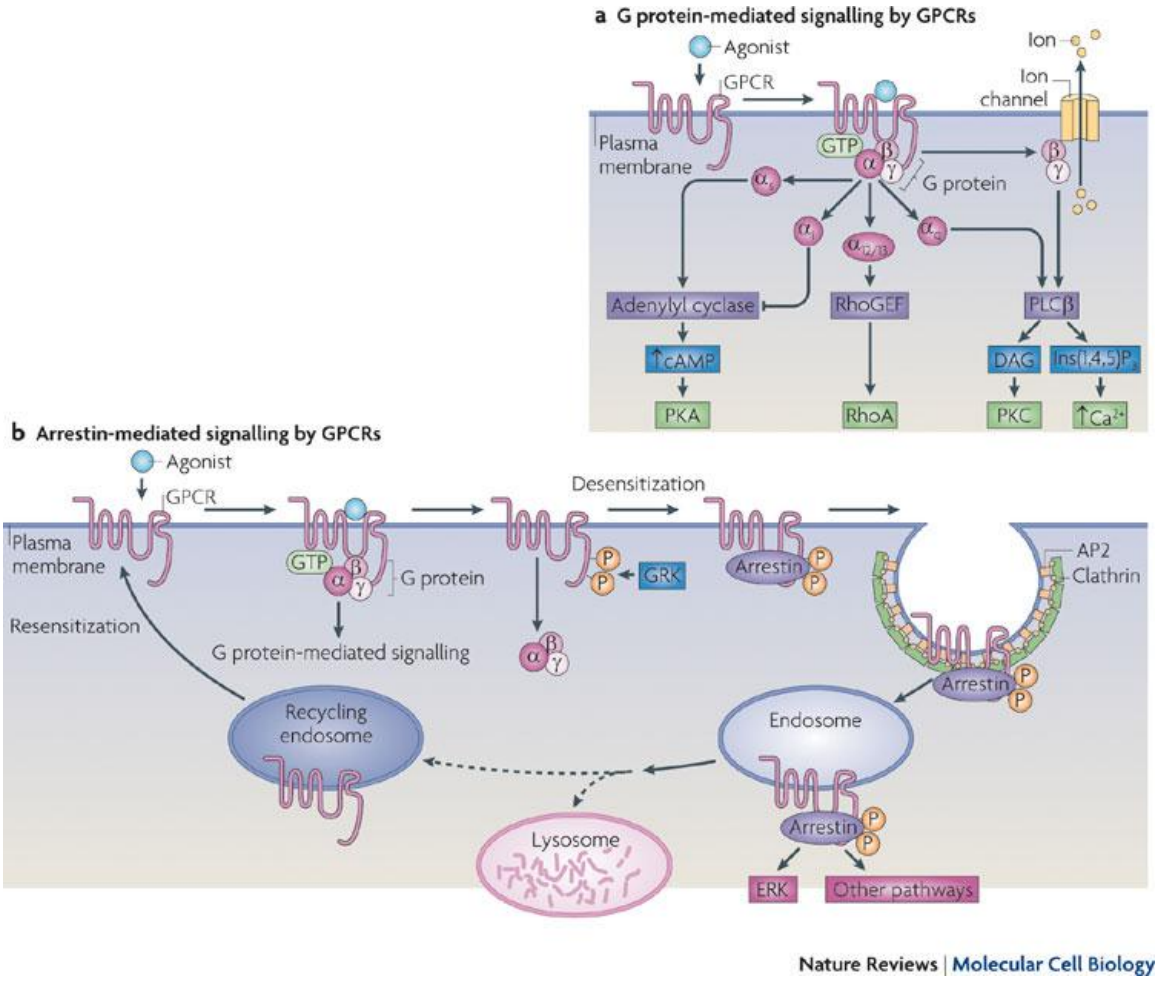

Figure 1.2. Canonical mechanisms of G protein-coupled receptor signaling. Overview of G protein-mediated **(**A**)** and arrestin-mediated **(**B**)** classical downstream signaling of G protein-coupled receptors (GPCRs).

1.7. Metabotropic Glutamate Receptors

1.7.A. Overview

The initial evidence that glutamate could activate signaling pathways independent from ionotropic glutamate receptors emerged in the late 1980s with studies of glutamate and quisqualic acid on isolated striatal neuron populations (Sladeczek et al., 1985), hippocampal neuronal populations in slice (Nicoletti et al., 1986) and studies in *Xenopis laevis* oocytes expressing brain mRNAs (Sugiyama et al., 1987). However, it was not until the early 1990s, that the first metabotropic glutamate receptor (mGluR) was cloned in the lab of Nakanishi in 1991 (Masu et al., 1991) and a few months later independently cloned by Houamed and colleagues (Houamed et al., 1991). By the mid-1990s, eight different metabotropic glutamate receptors had been identified, which could be grouped based upon sequence homology, agonist selectivity, and downstream coupling to Gα proteins (Pin and Duvoisin, 1995) comprising mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, and mGluR8. Metabotropic glutamate receptors within the same group show about 70% sequence identity (Conn and Pin, 1997).

The classifications of G protein-coupled receptors has evolved overtime, with the most recent grouping yielding 5 different families based on phylogenetic analysis and metabotropic glutamate receptors constituting the name-sake of one of these families, the glutamate family (Fredriksson et al., 2003). The glutamate receptor family (also known as Family C GPCRs) contains calcium sensing receptors (Ruat et al., 1995), metabotropic GABA-B receptors (Kaupmann et al., 1997), and taste receptors (Adler et al., 2000; Fredriksson et al., 2003; Kniazeff et al., 2011). The hallmarks of this family include a large extracellular N-terminus, heptahelical transmembrane spanning region, and large intracellular C-terminus. Additionally, most of these receptors predominantly couple via the second intracellular loop to G proteins, instead of via the third intracellular loop like most Rhodopsin/Family A GPCRs (Gomeza et al., 1996; Pin et al., 1994).

Metabotropic glutamate receptors have a large extracellular amino terminus with a Venus Fly Trap (VFT) domain that is similar to the periplasmic bacterial leucine/isoleucine/valine binding protein (LIVBP) (O'Hara et al., 1993), which was initially confirmed via X-ray crystallography studies of the mGluR1 N-terminus (Kunishima et al., 2000; Tsuchiya et al., 2002) (see Figure 1.2). The VFT domain is comprised of two lobes, each made of alpha helices and one β sheet, in which a pocket between the two lobes is for glutamate binding. This domain is then linked via a cysteine rich domain (CRD) to the seven transmembrane spanning (7TM) region or heptahelical domain. The structure of the cysteine rich domain complexed with the VFT domain was solved for mGluR3 and shown to contain two different subdomains of β sheet tetramers that are linked via a disulfide bond (Muto et al., 2007). The authors speculated that the CRD might be instrumental in the transduction of ligand binding in the VFT to biophysical sensing by 7TM and ultimately G protein activation (Muto et al., 2007).

Metabotropic glutamate receptors can also form functional dimers, thereby adding to the complexity of their signaling and function. Using FRET-based approaches, it was shown in 2008 that members of the Glutamate Family (Family C) GPCRs, including metabotropic glutamate receptors, can form receptor homodimers, but not oligomers, that can be detected at the cell surface (Maurel et al., 2008). More recently, it was also shown that metabotropic glutamate receptors can form heterodimers within their specific groups (intra-dimerization), while members of the Group II and Group III mGluRs can also form heteromers (inter-dimerization) (Doumazane et al., 2011).

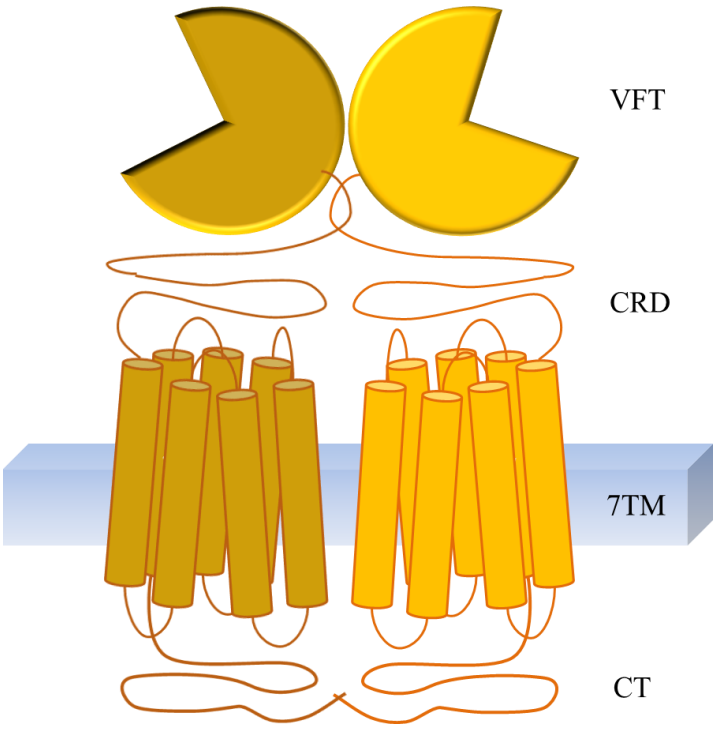

Figure 1.3. Schematic of key structural domains of metabotropic glutamate receptors. Over the past ten years, the crystal structures of various extracellular domains within the metabotropic glutamate receptors (mGluR) family have been solved. Depicted are two metabotropic glutamate receptors, representing homodimers, or heterodimers within the same class, which are thought to be the main population state for mGluRs. Glutamate binds between the two lobes of the venus fly-trap (VFT) extracelluar domain, which is linked via the cysteine-rich domain (CRD) to the 7 transmembrane spanning (7TM) or hepthahelical domain. The CRD domain is stablizied by many disulfide bonds. The intracellular portion of the mGluRs includes various intracelular loops linking the transmembrane spanning alpha helices, as well as an intracellular C-terminal domain. Across the mGluRs, much of the diversity occurs within the intracellular C-terminal domain. As a member of the glutamate suprafamily of GPCRs or the Family C GPCRs, Gα proteins are thought to couple predominantly via the second intracellular loop.

Group I Metabotropic Glutamate Receptors

Group I mGluRs, comprising mGluR1 and mGluR5, were identified in the early 1990s, with subsequent studies also sequencing and identifying a number of mGluR splice variants that primarily differed in the length of their C-terminal regions (Abe et al., 1992; Houamed et al., 1991; Laurie et al., 1996; Mary et al., 1997; Masu et al., 1991; Minakami et al., 1993; Minakami et al., 1994; Pin et al., 1992; Tanabe et al., 1992). For example, four mGluR1 splice variants, mGluR1a, mGluR1b, mGluR1c, and mGluR1d have been identified. The latter three variants have shortened intracellular C-terminal domains, relative to mGluR1; given that the C-terminus of receptors is often a site for protein-protein interactions suggests that the mGluR1 subtypes can differ in their C-terminal interacting partners. Similarly, mGluR5b has a 32 amino acid insertion in its C-terminus, relative to mGluR5a (Joly et al., 1995). However, mGluR5d is actually 267 amino acids shorter than mGluR5a, with the major difference resulting in a shorter C-terminus (Malherbe et al., 2002). In sum, the various Group I mGluR splice variants support the notion that the intracellular C-terminus is a key site of differential regulation across the subtypes.

Group I mGluRs comprising mGluR1 and mGluR5 classically couple through $G\alpha_q$ to activate phospholipase C (PLC), leading to the production of diacylglycerol (DAG) and inositol triphosphates (IP_3) and the subsequent release of intracellular calcium stores and activation of protein kinase C (PKC) (Figure 1.3 A). Moreover, Group I mGluRs can also modulate certain calcium and potassium channels, as well as activate ERK and AKT protein kinases (Ferraguti et al., 2008; Ribeiro et al., 2010). In contrast, there is some evidence that both mGluR1 and mGluR5 can signal to activate adenylyl cyclase in certain instances, thereby implicating a possibility for these receptors to couple to Gαs (Ferraguti et al., 2008). The EC_{50} for glutamate on mGluR1 (rat) or mGluR5 (rat) is around 8 or 9 μM, although it may be slightly higher for human receptors, as in the case of mGluR1 (see Table 1.1).

Group I mGluRs are meaningful players in synaptic plasticity and can also modulate ionotropic glutamate receptor function. KO mouse studies for Grm1 (the gene encoding mGluR1) or Grm5 (the gene encoding mGluR5) have revealed the importance of these receptors in mediating various aspects of synaptic plasticity, including long-term depression and long-term plasticity for mGluR1 or mGluR5, respectively (Aiba et al., 1994; Conquet et al., 1994; Lu et al., 1997). Grm1 KO mice also have motor impairments (Aiba et al., 1994; Conquet et al., 1994), and ectopic expression of mGluR1 in mouse melanocytes can induce melanoma formation, speaking to the significance of these receptors outside of the CNS (Ohtani et al., 2008). Grm5 KO mice fail to self-administer cocaine and do not display the typical increase in locomotor activity following cocaine exposure, supporting a role for mGluR5 in mediating behavioral responses to cocaine (Chiamulera et al., 2001). Additionally, mGluR5 is a therapeutic target for many neurological disorders including Fragile X disorder, in which chronic mGluR5 inhibition can correct the Fragile X phenotype in a Fmr1 KO mouse (Michalon et al., 2012). Inhibition of mGluR5 has also been proposed as a treatment strategy for Parkinson's disease, as assessed with chronic treatment of an mGluR5 antagonist in a non-human primate model of Parkinson's disease (Masilamoni et al., 2011). More recently, both mGluR1 and mGluR5 have also emerged as Huntington's disease-related genes (Kalathur et al., 2012), and their downstream signaling pathways have been implicated in striatal neuron survival (Ribeiro et al., 2010).

	mGluR1a	*mGluR5a*	*mGluR2*	*mGluR3*	*mGluR4a*	*mGluR6*	*mGluR7*	*mGluR8*	*Spec.*
Glutamate	-	-	-	-	-	-	-	0.02	Ms.
	0.9-21; 9	3-11; 8	0.3-20; 10	2-5; 4	3-17; 9	5-38; 20	1000-2500; 1800	3-10; 7.25	Rat
	3-184; 71	1-8; 3.7	N/A; 5	N/A; 400	13-38; 24	N/A; 7	56-640; 348	N/A; 11	Hu.
DHPG*	6-60; 24	N/A; 2	-	-	>1000	-	>1000	>1000	Rat
	10-374; 189	17-20; 19	-	-	>1000	-	>1000	>1000	Hu.
DCG-IV	N/A; >1000	510; 389-630	0.3-0.4; 0.4	0.09-.2; 0.1	N/A; 23	N/A; 40	N/A; 40	N/A; 32	Rat
	-	-	-	-	-	-	-	-	Hu.

Table 1.1. Summary of potencies (EC_{50}/IC_{50} values in µM) of several compounds acting on metabotropic glutamate receptors (mGluRs) 1-8. Values are assembled from all EC_{50} (black) or IC_{50} (red) values reported in (Schoepp et al., 1999) and exclude binding data. Table entries represent range; mean. Dashes represent information that was not available; ranges were not determined when only one value was available and were expressed as N/A. *DHPG has also been reported to activate NMDA receptors at concentrations of ≥ 3 µM. Abbreviations used: Ms. Mouse; Hu. Human.

Group II Metabotropic Glutamate Receptors

Group II mGluRs, comprising mGluR2 and mGluR3, were identified in the early 1990s, with splice variants for mGluR3 having been reported, but not functionally characterized. The most common mGluR3 splice variant lacks exon 4, which encodes an mGluR3 receptor that lacks the 7TM, but appears capable of trafficking to the plasma membrane (Sartorius et al 2006). Interestingly, this variant, along with two other GRM3 splice variants, was identified in the human brain. To date, it is unclear the function of this transcript variant is unknown.

Group II mGluRs are thought to classically couple to $G\alpha_{i/o}$ to directly inhibit adenylyl cyclase and prevent intracellular cAMP accumulation (Prezeau et al., 1994; Tanabe et al., 1992; Wroblewska et al., 1997). Engagement of activated $G\alpha_{i/o}$ with adenylyl cyclase leads to its inhibition and a subsequent reduction in intracellular cAMP, which indirectly prevents activation of protein kinase A (PKA) (Figure 1.3A). Activated $G\beta\gamma$ subunits can also activate G protein-inward rectifying potassium channels, GIRKs, or inhibit voltage sensitive N-type calcium channels, (Conn and Pin, 1997; Pin and Duvoisin, 1995) which can function to prevent neurotransmitter release from pre-terminal neurons (Schoepp, 2001). As shown in Table 1.1, the EC_{50} for the Group II mGluRs is relatively low (rat mGluR2 is 10 μM, rat mGluR3 is 4 μM), which would be within the range of extrasynaptic glutamate concentrations and further speaks their ability to regulate neurotransmitter release from terminals. Group II mGluRs have also been shown to signal through the kinase ERK in heterologous expression systems (Iacovelli et al., 2009) and to the kinases ERK and AKT in cultured astrocytes (Ciccarelli et al., 2007; D'Onofrio et al., 2001).

Activation of Group II mGluRs has been shown to inhibit release of glutamate from pre-terminal axons, in which the Group II mGluRs can function as autoreceptors to regulate the tone of synaptic glutamatergic activity (Schoepp, 2001). Moreover, activation of Group II mGluRs in co-cultures of neurons and astrocytes has been demonstrated to facilitate neuroprotection of

neurons challenged with NMDA treatment (Bruno et al., 1998; Bruno et al., 1997; Corti et al., 2007a; D'Onofrio et al., 2001; Kingston et al., 1999). Elegant studies in which co-cultures of neurons and astrocytes that were prepared from various combinations of either wild-type (WT), GRM2 (mGluR2) KO or GRM3 (mGluR3) KO mice subsequently revealed that the neuroprotective effects were primarily mediated via activation of astrocytic mGluR3 (Corti et al., 2007a).

Genetic and pharmacological studies have indicated that Group II mGluRs may be meaningful for a number of neuropsychiatric disorders including anxiety (Swanson et al., 2005) and schizophrenia. For example, systemic administration of Group II mGluR agonists are anxiolytic in mice, as behaviorally assessed via the elevated plus maze and open-Y maze (Helton et al., 1998; Linden et al., 2005; Monn et al., 1997) or with fear-potentiated startle in humans (Grillon et al., 2003). Group II mGluRs have also emerged as a therapeutic target for schizophrenia. Stage II clinical trials for a Group II mGluR agonist have demonstrated antipsychotic efficacy similar to an established schizophrenia drug, albeit with reduced side effects (Patil et al., 2007). Although some studies suggest mGluR2 may be primarily responsible for the anti-psychotic effects of the Group II mGluR drugs (Gonzalez-Maeso et al., 2008; Rorick-Kehn et al., 2007) several single nucleotide polymorphisms in GRM3, the gene encoding human mGluR3, are linked to schizophrenia (Sartorius et al., 2008) and altered dimerization of mGluR3 is seen in post-mortem schizophrenic brains (Corti et al., 2007b). Group II mGluRs have been shown to be a possible therapeutic target in glioblastoma multiforme (GBM), (Arcella et al., 2005; D'Onofrio et al., 2003) and in particular, GRM3 is a gene that is significantly upregulated in the neural classification of GBM, relative to the other three GBM classifications (Verhaak et al., 2010).

Group III Metabotropic Glutamate Receptors

The Group III mGluRs were also cloned in the 1990s, with mGluR4 being the first identified in 1992 (Tanabe et al., 1992). One splice variant of mGluR4 has been identified termed mGluR4b, which is missing the last 64 amino acids of mGluR4a in exchange for 135 different amino acids (Thomsen et al., 1997). The metabotropic glutamate receptor 6 was identified via isolation of cDNAs from a rat retinal library by Nakajima et al. (Nakajima et al., 1993). To date, there are no mGluR6 splice variants. The cloning of mGluR7 was in 1994 (Okamoto et al., 1994) with independent identification coming two months later from a rat olfactory bulb library (Saugstad et al., 1994). Subsequent studies identified additional mGluR7 splice variants termed mGluR7b-e, (Corti et al., 1998; Flor et al., 1997; Nicoletti et al., 2011). Rat mGluR8 was cloned and identified in 1997 and shown to be pertussis toxin (PTX) sensitive via PTX-mediated prevention of glutamate-induced GIRK responses in *Xenopis laevis* oocytes (Saugstad et al., 1997). One mGluR8 splice variant has been identified, termed mGluR8b (Corti et al., 1998).

Like Group II mGluRs, Group III mGluRs, comprising mGluR4, mGluR6, mGluR7, and mGluR8 also classically couple to $G\alpha_{i/o}$ to inhibit adenylyl cyclase (Duvoisin et al., 1995; Nakajima et al., 1993; Saugstad et al., 1994; Tanabe et al., 1992; Tanabe et al., 1993), although mGluR8 is reportedly a "weak coupler" to adenylyl cyclase inhibition (Saugstad et al., 1997). Furthermore, the $G\beta\gamma$ subunits of Group III mGluRs have been shown to activate G protein inward rectifying potassium channels (GIRKs) (Saugstad et al., 1997; Saugstad et al., 1996; Sharon et al., 1997). Group III mGluR activation can also lead to the inhibition of neurotransmitter release (Cochilla and Alford, 1998). Similar to Group II mGluRs, activation of Group III mGluRs has also been shown to be neuroprotective, in particular mGluR4 is meaningful in rescuing neurons challenged with NMDA treatment (Bruno et al., 2000).

Studies of Group III mGluR-deficient mice have implicated these glutamate receptors in a variety of disease states, including epilepsy (Bertaso et al., 2008; Sansig et al., 2001) and

anxiety (Duvoisin et al., 2005; Linden et al., 2002), as well as being salient for physiological responses such as ON-responses of retinal bipolar cells to visual stimuli (Masu et al., 1995)] or working memory (Holscher et al., 2004). Interestingly, as shown in Table 1.1, an average of the reported EC_{50} for glutamate on rat mGluR7 is approximately 1800 μM or on human mGluR7 348 μM (Conn and Pin, 1997; Schoepp et al., 1999), which is significantly higher than the other receptor subtypes (Table 1.1). Along with the susceptibility to epilepsy phenotype, this implies mGluR7 may play a key role during times of high synaptic activity and high extracellular glutamate concentrations (Nicoletti et al., 2011).

1.7.B. Distribution

Group I mGluRs

Group I mGluRs are widely distributed throughout the adult brain and are also abundant in the developing brain. The metabotropic glutamate receptor 1 has been found in Purkinje cells in the cerebellum and in mitral cells in the olfactory bulb, as well as in neuropil in the striatum (SNpc and GP) and in thalamic relay nuclei (Baude et al., 1993; Martin et al., 1992; Shigemoto et al., 1992). Immunolabeling for mGluR5 has been found throughout the adult rat brain, with highest labeling found in olfactory bulb, caudate/putamen, lateral septum, cortex, and hippocampus (Romano et al., 1995). In non-human primate models, Group I mGluR labeling has been found in the prefrontal cortex (Muly et al., 2003), as well as mGluR5 labeling in the striatum (Bogenpohl et al., 2012).

At the ultrastructural level, Group I mGluRs are predominantly found in post-synaptic elements such as dendrites or spines in rodent and non-human primate studies, and to a lesser extent on axon terminals and unmyelinated axons (Baude et al., 1993; Mitrano and Smith, 2007; Muly et al., 2003; Romano et al., 1995). Glial labeling for Group I mGluRs has also been found, although mGluR5 is the predominant glial Group I mGluR (Mitrano and Smith, 2007). In

monkey pre-frontal cortex, both mGluR1 and mGluR5 are predominantly in post-synaptic elements, such as dendrites and spines; however, both subtypes can also be found in pre-terminal elements, such as unmyelinated axons or axon terminals that form asymmetric and symmetric synapses, implying that Group I mGluRs can also be found in interneuron populations (Muly et al., 2003). The various Group I mGluR transcript variants have differential expression patterns, with mGluR1a being predominant subtype over mGluR1b and mGluR1c in the adult brain (Ferraguti et al., 1998; Pin et al., 1992). Interestingly, it was found in comparing young versus old rats that although mGluR1 and mGluR5 are abundant in unmyelinated axons from young rodent SNpc tissue, their expression is lost into adulthood (Hubert and Smith, 2004). In contrast, only mGluR1 glial expression is lost in the SNpc, while mGluR5 expression is reduced but not eliminated entirely. This implicates Group I mGluRs in the developing brain and highlights a presynaptic role in this particular brain region (Hubert and Smith, 2004). However, of the mGluR5 splice variants, mGluR5b appears to be more highly expressed in adulthood, including in neurons and glia (Romano et al., 1996; van den Pol et al., 1995). More recently, it was shown that glial mGluR5 expression is lost in mouse cortex and hippocampus after 3 weeks of post-natal development, suggesting that glial mGluR5 has a strictly early-on developmental role (Sun et al., 2013).

Group II mGluRs

Like Group I mGluRs, Group II mGluRs are distributed widely throughout the brain, as *in situ* hybridization and immunohistochemical studies in rodents have revealed that Group II mGluRs are abundant in the cerebral cortex, hippocampus, and olfactory bulb, with some staining in the striatum, nucleus accumbens and thalamic nuclei, as well as intensive labeling within the cerebellum (Fotuhi et al., 1994; Muly et al., 2007; Neki et al., 1996; Ohishi et al., 1994; Petralia et al., 1996; Tamaru et al., 2001; Tanabe et al., 1993; Testa et al., 1994). Few studies have been

done in humans (Blumcke et al., 1996), although in early 2000, Phillips et al. reported Group II mGluR immunoreactivity in human medial temporal lobe cortex (Phillips et al., 2000).

At the cellular and subcellular level, Group II mGluRs are abundant in axons and glia (Sun et al., 2013), with some expression in post-synaptic dendrites and spines that appears to vary across brain regions, although until recently, little quantification had been performed of the cellular and subcellular localization of Group II mGluRs. Specifically, immunolabeling of Group II mGluRs in regions CA1 and CA3 of hippocampus was found in glial elements and mossy fibers (axons) from granule cell neurons (Petralia et al., 1996), which based upon mRNA studies was presumed to be glial mGluR3 labeling and neuronal mGluR2 and mGluR3 labeling respectively (Petralia et al., 1996). Out of the regions examined including cerebral cortex, hippocampus, and caudate/putamen, Group II mGluR immunolabeling was observed in dendrites, unmyelinated axons, and glia, although its distribution was not specifically quantified. Qualitatively speaking, the reported dendritic immunolabeling by Petralia et al. was more pronounced in the granule cell layer of the hippocampus, where mRNAs for mGluR2 and mGluR3 have been observed to be highly expressed, relative to the cortex and caudate/putamen which was more lightly labeled (Petralia et al., 1996). Petralia et al. also argued that "some of the most prominent and widespread staining seen in the ultrastructural studies" was of glial processes in the cerebral cortex, hippocampus, and caudate/putamen, which they presumed to be mGluR3 (Petralia et al., 1996). Cerebellar labeling with a C-terminal Group II mGluR antibody was found in axon terminals, dendrites, and in neuronal cell bodies of Golgi cells, as well as in glia processes (Ohishi et al., 1994). Staining was predominantly in the granular layer of the cerebellar cortex (Ohishi et al., 1994).

Additionally, in the dentate granule layer of the hippocampus in the adult mouse, studies using an mGluR3-selective antibody in mGluR2 KO mice revealed immunolabeling for mGluR3 in spines, axon terminals, and glial processes, although its relative distribution across elements

was not quantified (Tamaru et al., 2001). One caveat to these studies is that it is unclear whether mGluR3 could be compensatorily upregulated in mGluR2 KO mice.

Group III mGluRs

Relative to Group I and Group II mGluRs, Group III mGluRs are not as widely distributed in the central nervous system. Overall, mGluR4 is highly abundant in the cerebellum with some detection in the basal ganglia, sensory relay nuclei of the thalamus, the dentate molecular layer of the hippocampus (Corti et al., 2002), and olfactory bulb (Kinoshita et al., 1996). More recently, analysis of the expression of mGluR4 in the non-human primate basal ganglia has been characterized and found to be predominantly pre-synaptic in the globus pallidus externa (GPe) and increased in its post-synaptic distribution in the striatum (Bogenpohl et al., 2013). In contrast, mGluR7a is more widely distributed across the central nervous system and in rodent studies has been found in the piriform cortex, superior colliculus, thalamic reticular nucleus, hippocampus, substania nigra pars reticulata, locus coeruleus and cerebellum, although its abundance in these regions can vary significantly across development (Bradley et al., 1998; Kinoshita et al., 1998), while mGluR7b is more restricted and found in hippocampus, globus pallidus, and ventral pallidum (Kinoshita et al., 1998). Expression of mGluR8 has been detected in the lateral perforant pathway in the dentate gyrus and CA3 region of the hippocampus (Shigemoto et al., 1997). Additionally, analysis of the mRNA distribution of mGluR8 in rodents has revealed it to be abundant in the pontine nucleus and reticulotegmental nucleus of the pons, with lesser expression in the olfactory bulb, cortex and thalamic reticular nucleus (Corti et al., 1998). Finally, immunohistochemical studies have demonstrated that mGluR6 is exclusively expressed in the outer plexiform layer of the retina, whereas the other remaining Group III mGluRs, mGluR4, mGluR7, and mGluR8 are expressed in the inner plexiform layer, implicating Group III mGluRs in the regulation of retinal light responses (Quraishi et al., 2007).

In general, Group III mGluRs are located on presynaptic nerve terminals (Bogenpohl et al., 2013; Bradley et al., 1998; Corti et al., 2002; Kinoshita et al., 1996; Kinoshita et al., 1998; Shigemoto et al., 1997; Shigemoto et al., 1996); and are localized proximal to the active release zone, relative to Group II mGluRs (Shigemoto et al., 1997; Wada et al., 1998). Localization of mGluR4 has been shown at striatopallidal GABAergic terminals in both the rodent (Corti et al., 2002) and monkey (Bogenpohl et al., 2013), where they are presumed to function as heteroreceptors whose activation can prevent the release of GABA from presynaptic terminals. Likewise, mGluR7 immunoreactivity has been observed on GABAergic terminals of striatal projection neurons (Corti et al. 2002), as well as in interneurons in the hippocampus, where it can presumably function as a heteroreceptor to inhibit GABA release, upon stimulation glutamate.

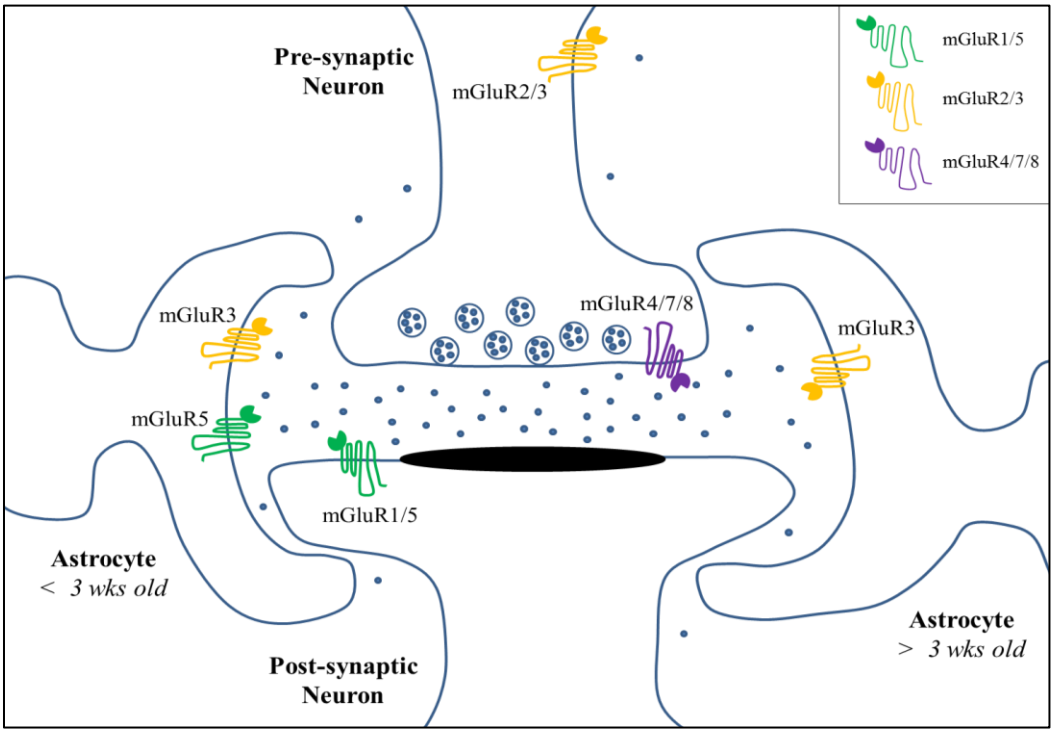

Figure 1.4. Overview of ultrastructural localization of mGluRs in the central nervous system. A hypothetical asymetric synapse is shown in which a preterminal axon loaded with glutamatergic vessicles is forming an asymetric synapse onto a spine, which contains a post-synaptic specialization known as the post-synaptic density or PSD. Astrocytic end-feet, termed perisynaptic astrocyte processes or PAPs, are forming tripartite synapses with the pre- and post-synaptic element and are effectively covering the synaptic space. All three metabotropic glutamate receptor familys are uniquely distributed at the tripartite synapse. Group I mGluRs, containing mGluR1 and mGluR5 (green) are predominatly expressed in post-synaptic spines and dendrites and tend to belocalized perisynaptic to the PSD specialiation. Expression of mGluR5 can also be found on glia, which are presumed to be astrocytes when they localize at the synapse. Moreover, glial mGluR5 expression declines shortly after birth and is significantly down-regulated in adult rodents and human tissue. Group II mGluRs, containing mGluR2 and mGluR3

are generally expressed on presynaptic elements and are localized on pre-terminal, unmyelinated axons (gold) where they can respond to extrasynaptic concentrations of glutamate released from their concentric synapse or can respond to glutamate that is released from hetero-synapses. Of the two Group II mGluRs, mGluR3 is predominantly expressed in glia. Group III mGluRs, containing mGluR4,7,8 and also tend to be localized on presynaptic elements in the cerebrum and cerebellum (purple), while mGluR6 is exclusively localized in the retina. The group III mGluRs are located near the active release sites, and mGluR7 in particular has been shown to localize at this site. The differences in the distribution of the mGluRs is also reflected in their varying affinities for glutamate. For example, mGluR7 has the lowest affinity for glutamate, yet it is localized in the heart of the synapse where it can encounter the highest concentration of glutamate; whereas the Group II mGluRs, which have the highest affinity for glutamate, are located distal to the glutamate release site and thus encounter a low glutamate concentrations.

1.8. NAAG: A putative mGluR3-selective endogenous agonist

N-acetylaspartylglutamate (NAAG) is a small neuropeptide that has been reported to be an mGluR3-specific endogenous agonist, as recently reviewed by (Neale et al., 2011). As shown in studies of Chinese Hamster Ovary (CHO) or Human Embryonic Kidney (HEK293) cells overexpressing the various mGluR subtypes, only mGluR3 was activated by NAAG, as evidenced by measured inhibition of forskolin-stimulated adenylyl cyclase responses (Wroblewska et al., 1997). Although it has been pointed out that certain commercially-available preparations of NAAG have been contaminated with glutamate (Chopra et al., 2009; Fricker et al., 2009), the Neale lab has argued that much of the work on the pharmacological and physiological actions of NAAG on mGluR3 have been done using NAAG preparations that have been repurified within their lab and are thus not contaminated with glutamate (Neale et al., 2011). Neale and colleagues have also put forth an alternative hypothesis arguing that the failure of purified NAAG to elicit activation of G protein-inward rectifying potassium channels in cells co-expressing mGluR3 could instead support the notion that glutamate and NAAG can activate distinct pathways downstream of receptor activation (Neale et al., 2011), a concept known as biased agonism (Urban et al., 2007; Violin and Lefkowitz, 2007). Furthermore, Neale and colleagues have also argued that studies of NAAG have been complicated due to its rapid inactivation by glutamate carboxypeptidases II and III (also known as N-acetylated alpha-linked acidic dipeptidase, NAALADase), which can in turn catabolize NAAG into N-acetylaspartate (NAA) and glutamate (Bzdega et al., 1997; Carter et al., 1996).

Additionally, NAAG has also been reported to have mixed actions at the NMDA receptor, with published studies reporting that NAAG can act as a weak agonist (Kolodziejczyk et al., 2009; Westbrook et al., 1986), an antagonist (Bergeron et al., 2005; Bergeron et al., 2007), or have no effect (Fricker et al., 2009; Losi et al., 2004). Given that the concentrations of NAAG used in the studies supporting its role as an agonist were relatively high ($\geq 300\ \mu M$), implies that

it is unlikely that NAAG is an NMDA agonist at physiological concentrations (Neale et al., 2011). However, recently it has still been argued that NAAG is an antagonist at NMDA receptors, as well as an mGluR3-selective agonist (Bergeron and Coyle, 2012). The role of NAAG as an endogenous mGluR3 biased agonist and source of extracellular glutamate remains an intriguing area of research that will be important for understanding mGluR3 function.

1.9. Additional Excitatory Amino Acids: D-serine and D-aspartate

Besides L-glutamate, additional amino acids have been found within the mammalian CNS that are thought to function as more than just amino acids. The two most extensively-studied amino acids include D-serine (Mothet et al., 2000; Snyder and Kim, 2000) and D-aspartate (D'Aniello, 2007; D'Aniello et al., 2011; Kim et al., 2010; Schell et al., 1997). D-serine acts as a co-factor for NMDA receptors and occupies the glycine binding site (Mothet et al., 2000). D-serine can be released from astrocytes as a gliotransmitter. D-aspartate can be generated via the conversion of L-aspartate to D-aspartate via aspartate racemase (D'Aniello et al., 2011; Miao et al., 2006), which is presumed to occur within the soma, whereby D-aspartate is then trafficked to the axon terminal and packaged into vesicles for calcium-mediated release. Although D-serine has been shown to bind to the NMDA receptor, the receptor target for D-aspartate is less understood. Binding studies with cold and hot versions of various excitatory amino acid enantiomers indicated that D-aspartate has a receptor target that is distinct from a glutamate receptor target (D'Aniello et al., 2011), although as this target is currently not known, this is still controversial. It has been reported that D-aspartate can act as a low-affinity competitive antagonist for AMPA receptors in hippocampal neurons or *Xenopus laevis* oocytes expressing recombinant AMPARs, which is similar to the ability of NMDA to inhibit AMPARs (Gong et al., 2005).

1.10. PDZ Proteins[2]

Receptor function is often regulated by receptor-interacting proteins, which can profoundly influence receptor signaling, trafficking, and/or pharmacology (Ritter and Hall, 2009). One of the most well-studied classes of receptor-interacting partners is the family of PDZ domain-containing scaffold proteins. PDZ domains are specialized protein–protein interaction modules, which derive their name from the first three proteins in which they were identified: the postsynaptic density protein of 95 kDa (*P*SD95), the *Drosophila* protein discs-large tumor suppressor A (*D*lgA), and the tight junction protein zona-occludens 1(*z*o-1). PDZ domains are approximately 90 amino acids in length and typically recognize target motifs at the extreme C terminus of interacting proteins (Sheng and Sala, 2001), although some PDZ proteins can bind to an internal PDZ ligand not found at the extreme C terminus (Hillier et al., 1999). Most PDZ-interacting proteins possess a C-terminal motif consisting of a hydrophobic amino acid at the terminal position and either a hydrophobic amino acid, a hydroxyl-bearing amino acid (S or T), or an acidic amino acid (D or E) at the −2 position (Stiffler et al., 2007).

PDZ domain-mediated interactions are often of very high affinity and therefore amenable to detection by a number of different screening approaches. For example, many receptor/PDZ interactions have been first detected in yeast two-hybrid screens (Fields and Song, 1989). Other screening approaches include phage display (Bair et al., 2008), fluorescence polarization (Park and Raines, 2004), and pull-down studies from tissue samples followed by mass spectrometry (Brymora et al., 2004).

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[2] This section has been reproduced from Ritter SL and RA Hall (2011) Detection and characterization of receptor interactions with PDZ domains. *Methods Mol Biol* **756:** 345-356 with permission from Signal Transduction Protocols, © Springer Science+Business Media, LLC 2011. Minor edits have been made to this section.

Receptor	Species	Gen Bank AN	Amino Acids	CT Residues	Class
GLAST	Rat	NP_062098.1	543	ETKM	I
GLT1c	Rat	AAS89003.1	145	QSWV	I
EAAC1	Rat	AAB09773.1	523	TSQF	I
EAAT4	Rat	AAB72086.1	561	ESVM	I
EAAT5	Rat	NP_001102443.1	570	ETNV	I
mGluR1a	Rat	NP_058707.1	1199	SSTL	I
mGluR1b	Rat	NP_001107802.1	906	HAQL	NC
mGluR2	Rat	NP_001099181.1	872	TSSL	I
mGluR3	Rat	NP_001099182.1	879	TSSL	I
mGluR4a	Rat	NP_073157.1	912	NHAI	III
mGluR4b	Rat	AAA88788.1	983	GDGL	III
mGluR5a	Rat	NP_058708.1	1171	SSSL	I
mGluR5b	Rat	ACB45671.1	1203	SSSL	I
mGluR6	Rat	NP_075209.1	871	EDAK	NC
mGluR7a	Rat	NP_112302.1	915	NLVI	II
mGluR7b	Human*	NP_870989.1	922	PPTV	II
mGluR8a	Rat	NP_071538.1	908	NHSI	III
mGluR8b	Rat	CAA72040.1	19	GSTS	NC

Table 1.2. Distribution of C-terminal type 1 PDZ-interacting motifs on glutamate transporters and metabotropic glutamate receptors. Genbank accession numbers were compiled from NCBI databases. The total number of amino acids are listed and the last four amino acids of the protein. PDZ domains are classified as Type 1, Type II, Type III, or no-classification (NC). * Rat not available. This table has been adapted and updated from Maryse Paquet's dissertation published in 2005.

1.11. NHERF Proteins

1.11.A. Overview

The NHERF protein family is a family of four PDZ scaffolding proteins that have been shown to bind and interact with a variety of transporters, G protein-coupled receptors, tyrosine kinase receptors and various cytosolic effectors (Ardura and Friedman, 2011). The first member of this family, NHERF-1, was identified in the mid-1990s by two different labs via independent approaches. The laboratory of Ed Weinman at the University of Maryland was searching for a co-factor that was required for protein kinase A- (PKA) mediated inhibition of the sodium proton exchanger isoform 3 (NHE3) in the kidney (Weinman et al., 1995) that they termed Na+ H+ Exchanger Regulatory Factor 1, NHERF-1. Around the same time, the Bretscher lab was searching for binding partners of the protein ezrin, a protein that was originally identified in microvilli as a bridge between the plasma membrane and the actin cytoskeleton, and subsequently identified ezrin-binding phosphoprotein of 50 kDa, or EBP50 (Reczek et al., 1997). In the late 1990s, the laboratory of Mark Donowitz used a yeast two hybrid screen to identify novel NHE3 binding partners and sequenced the closely related protein to identify E3KARP, or NHE3 Kinase A Regulatory Protein, which was later termed NHERF-2 (Yun et al., 1997). NHERF-1 (EBP50) and NHERF-2 (E3KARP, TKA-1, SIP-1) share 53% and 51% sequence identify in the mouse and human, respectively (BLASTp 12-6-12). In fact, NHERF-1 and NHERF-2 both share two tandem type PDZ domains and a C-terminal ERM binding domain that links to the actin-associated FERM proteins ezrin, radixin, moesin and merlin (Murthy et al., 1998; Reczek et al., 1997).

Two additional NHERF family members have been reported: NHERF-3, (Custer et al., 1997), also known as PDZK1, CAP70, or PDZ-dc-1 and NHERF-4 (Gisler et al., 2001), also known as PDZK2, or IKEPP. NHERF-1 shares 39% sequence homology with NHERF-3, and 40% sequence homology with NHERF-4 (Ensemble Blast p 12-6-12). Interestingly, NHERF-3

and NHERF-4 lack the C-terminal ERM binding domain and instead have four tandem PDZ domains, making them distinct from the first two NHERF family members (see Figure 1.5).

NHERF-1 and NHERF-2 have been shown to bind to a variety of exchangers, transporters, G protein-coupled receptors, tyrosine kinase receptors, and cytosolic proteins, via their PDZ domain or ERM interacting motifs (Ardura and Friedman, 2011; Weinman et al., 2006). Although NHERF-1 and NHERF-2 are distributed throughout tissues in the body, their levels are higher in kidney, small intestine, placenta, brain, lung, stomach, and heart (Ingraffea et al., 2002; Reczek et al., 1997). It is interesting to note that NHERF-1 appears to be particularly enriched in tissues with extensive, polarized epithelia, such as small intestine, kidney, liver, and placenta (Reczek et al., 1997).

Recently, a cholesterol recognition amino acid consensus, or CRAC, motif was identified in a number of proteins containing PDZ domains, with the PDZ domain of NHERF-1 serving as the representative example (Sheng et al., 2012). This domain was shown to mediate NHERF-1 binding to cholesterol-rich compartments of the plasma membrane, without disrupting the ability of NHERF-1 to bind to other interacting partners, like the Cystic-Fibrosis Transmembrane Conductance Regulator, or CFTR (Sheng et al., 2012). In screening 149 mouse PDZ domains, Sheng et al. did not identify NHERF-2 as also having this CRAC domain, thereby highlighting a potential difference between NHERF-1 and NHERF-2 that may have consequences for NHERF-mediated regulation of trafficking.

The significance of NHERF binding to its interacting partner can have many outcomes; however a few primary themes have emerged (Ritter and Hall, 2009). For most of its interacting partners, NHERF binding has been demonstrated to help stabilize its interacting partners at the plasma membrane, including its interaction with the kappa opioid receptor (KOR) to enhance receptor recycling (Li et al., 2002) and interaction with the parathyroid hormone receptor (PTH1R) to stabilize the receptor at plasma membrane independent of effects on recycling (Wang

et al., 2007). However, it was demonstrated for the $P2Y_{12}R$ that NHERF-1 interaction indirectly promoted receptor internalization, by acting as a scaffold that recruited arrestin to the receptor following agonist stimulation (Nisar et al., 2012). However, one could argue that because this model demonstrated that disengagement of NHERF1 with the receptor led to enhanced arrestin binding, it does not provide evidence against direct NHERF association with a receptor to enhance receptor stabilization.

Other themes concerning the ways in which NHERF proteins can interact with their partners includes modifying the G protein-dependent coupling for certain GPCR-interacting partners like the PTH1R (Wang et al., 2010), as well as organizing macromolecular complexes to direct the time course and/or intensity of receptor-mediated downstream signaling (Ritter and Hall, 2009).

1.11.B. NHERF-1/-2 Deficient Mice

NHERF-1 knockout (KO) mice were originally developed by the laboratory of Ed Weinman at the University of Maryland via homologous recombination of exon 1 in the NHERF-1 gene (Shenolikar et al., 2002). The mice were subsequently crossed onto a C57BL6/J background (JAX®); however certain groups (Ursula Seidler) have also crossed this background to FVB/N and performed studies comparing NHERF-1, NHERF-2, and NHERF-1/-2 KO mice (Singh et al., 2009). The initial reported phenotype of the NHERF-1 KO mice includes phosphate wasting, decreased bone mineral content, and increased urinary excretion of phosphate, presumably via enhanced internalization of the sodium-dependent phosphate transporter 2a (Npt2a) (Shenolikar et al., 2002). NHERF-1 KO mice also have defective cAMP-mediated regulation of the NHE3 in the kidney (Shenolikar et al., 2002). Additional studies also revealed that NHERF-1 deficient mice have increased urinary secretion of calcium and uric acid, in addition to the previously reported increase in phosphate secretion, which presumably may be

explained by the decreased apical membrane localization of the urate transporter 1, URAT1 (Cunningham et al., 2007). Finally, initial reports of the NHERF-1 KO mice (C57BL6/J background) revealed that some of the female NHERF-1 KO mice died from hydrocephaly, although this was not quantified and no additional CNS characterization was performed (Shenolikar et al., 2002). In published reports, NHERF-1 KO have been considered to be congenic on C57BL6/J, although a test of 32 separate alleles of the NHERF-1 KO mice that were recently acquired from JAX® identified 8 out of 32 SNPs that were not from C57BL6/J and were instead from 129S, the background used in the initial generation of the mice (JAX® Database).

NHERF-2 KO mice were originally developed by Lexicon Genetics via a retroviral gene trapping procedure for the laboratory of Hugo R. de Jonge and Boris M. Hogema (Broere et al., 2007). The reported phenotypes include alterations in the intestines of NHERF-2 KO mice such as shorter villi, deeper crypts, decreased epithelial number, which is accompanied by a decrease in the localization of the NHE3 in the brush border membrane (Murtazina et al., 2011), as well as enhanced forskolin-stimulated intestinal HCO_3^- secretion (Singh et al. 2007). The increased phosphate and uric acid secretion observed in the NHERF-1 KO mice are absent in NHERF-2 KO mice (Cunningham et al., 2008). There has been no CNS characterization of these mice.

The double NHERF-1/NHERF-2 KO mice have been reported on the FVB/N background (Broere et al., 2007; Singh et al., 2009) and display some alterations in the HCO3- secretions, relative to NHERF-1 KO mice. However, no CNS characterization has been reported for these mice.

1.12. Central Hypothesis

Given the prevalence of the type 1 PDZ interacting motifs on glutamate transporters and metabotropic glutamate receptors (Table 1.1), we hypothesize that the C-terminus of these proteins is a site of interaction with and regulation by PDZ proteins. To this end, we used the PDZ proteomic array developed within this lab, to screen the glutamate transporter and receptor C-termini against 96 distinct Type 1 PDZ domains. Interestingly, the lab has previously published that mGluR5 can interact with the PDZ protein NHERF-2 (Paquet et al., 2006a). Association of mGluR5 with NHERF-2 can enhance mGluR5 downstream signaling to intracellular calcium stores and can also promote mGluR5-mediated cellular toxicity. Therefore, a likely interacting partner for the glutamate transporters and metabotropic glutamate receptors are the NHERF proteins. Our goal was to examine putative PDZ-interacting partners for other mGluRs and glutamate transporters and then proceed to characterize any identified interactions.

CHAPTER 2

Glutamate Transporter Regulation by NHERF Proteins[3]

[3] Reproduced with permission from original publication: Ritter SL, Asay MJ, Paquet M, Paavola KJ, Reiff RE, Yun CC, Hall RA (2011) GLAST stability and activity are enhanced by interaction with the PDZ scaffold NHERF-2. *Neurosci Lett* **487:** 3-7. Substantial changes have been made to the text and some figures have been added.

2.1. Summary

The astrocytic glutamate transporter GLAST (also known as EAAT1) is a key regulator of extracellular glutamate levels in many regions of vertebrate brains. To identify novel interacting partners that might regulate the localization and function of GLAST in astrocytes, we screened the transporter's C-terminus (GLAST-CT) against a proteomic array of 96 different PDZ domains. The GLAST-CT robustly and specifically interacted with PDZ domains from two related scaffolding proteins, the Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF-1 and NHERF-2). Studies on cultured rat cortical astrocytes revealed that these cells are highly enriched in NHERF-2 relative to NHERF-1. Endogenous GLAST and NHERF-2 from cultured astrocytes were found to robustly co-immunoprecipitate, and further co-immunoprecipitation studies on mutant versions of GLAST expressed in transfected cells revealed the GLAST/NHERF-2 interaction to be dependent on the last amino acid of the GLAST-CT. Knockdown of endogenous NHERF-2 in astrocytes via siRNA treatment resulted in a significant reduction in GLAST activity, which corresponded to significantly reduced total expression of GLAST protein and reduced half-life of GLAST, as assessed in pulse-chase metabolic labeling studies. These findings reveal that NHERF-2 can interact with GLAST in astrocytes to enhance GLAST stability and activity.

2.2. Introduction

Astrocytic glutamate transporters are primarily responsible for clearing extracellular glutamate and are essential in regulating the amount of extracellular glutamate following high levels of synaptic stimulation, in order to prevent excitotoxic cellular death (Rothstein et al., 1996; Tanaka et al., 1997). Given their significant role in regulating glutamatergic neurotransmission, astrocytic glutamate transporters have emerged as therapeutic targets for a number of disorders of the central nervous system (Bunch et al., 2009; Dunlop, 2006; Rao and

Sari, 2012; Reissner and Kalivas, 2010). A significant focus of research endeavors has been to increase the expression of glutamate transporters, as loss of the astrocytic glutamate transporters, GLAST (EAAT1) and GLT-1 (EAAT2) via genetic KO approaches, can lead to extensive neurodegeneration in mice (Rothstein et al., 1996; Tanaka et al., 1997). On the other hand, overexpression or enhancement of glutamate transporter function can be beneficial. Transduction of rat brains with adeno-associated viral (AAV) vectors encoding GLT-1, prior to induction of ischemia and stroke via middle cerebral artery occlusion, can significantly reduce infarct volume size and associated cellular death, as well as promote behavioral recovery (Harvey et al., 2011). More recently, cultured astrocytes from a transgenic mouse (SOD1G93A) modeling Amyotrophic Lateral Sclerosis (ALS), demonstrated that upregulation of GLAST and GLT-1 in WT cultured astrocytes is critical to the observed protection of a motorneuron cell line, which is disrupted in the transgenic SOD1G93A mice in which the upregulation of the glutamate transporters is no observed (Benkler et al., 2013). In summary, significant interest exists in targeting expression levels of glutamate transporters as a therapeutic approach. In light of the Type 1 postsynaptic density protein of 95 kDa, disc large tumor suppressor A, and zona-occludens 1 (PDZ)-interacting motif on the extreme C-terminus of GLAST, we screened a comprehensive PDZ array consisting of 96 distinct Type I PDZ domains to identify novel GLAST-interacting partners. As PDZ proteins are known to scaffold their effectors and often increase their stability at the plasma membrane in neurons (Feng and Zhang, 2009), we predicted that any identified GLAST-PDZ scaffolds would similarly act as an stabilizing scaffold in glial organization.

2.3. Results

In order to gain a panoramic view of potential PDZ interactions for GLAST, we screened a GST fusion protein corresponding to the GLAST-CT against a previously-described PDZ proteomic array containing 96 distinct PDZ domains (Fig. 2.1). The GLAST-CT did not

detectably bind to the vast majority of PDZ domains on the array. However, strong binding of the GLAST-CT was observed to both PDZ domains of the Na^+/H^+ exchanger regulatory factor (NHERF-1), as well as to PDZ domain 2 of the related protein NHERF-2 (Fig. 2.1). Weaker binding was also observed to the first PDZ domain of NHERF-2. The interaction of GLAST with NHERF-1 has been reported previously (Lee et al., 2007a), but the association of GLAST with NHERF-2 is novel. Thus, we characterized the GLAST/NHERF-2 interaction in further detail.

Saturation binding assays overlaying His-tagged NHERF-2 PDZ2 onto purified GLAST-CT-GST revealed the K_D of the GLAST/NHERF-2 PDZ2 interaction to be 196 nM (Fig. 2.2A). This affinity is in the range of other PDZ domain-mediated associations that are known to be physiologically relevant (Sala et al., 2001). Furthermore, full-length GLAST from astrocyte lysates was robustly pulled down by GST-NHERF-2 (Fig. 2.2B).

In order to assess whether GLAST and NHERF-2 might associate in a cellular context, co-immunoprecipitation experiments were performed. Immunoprecipitation of endogenous NHERF-2 from cultured primary astrocyte lysates resulted in a robust co-immunoprecipitation of endogenous GLAST (Fig. 2.3A). We then examined whether GLAST/NHERF-2 cellular association was dependent on the PDZ-binding motif. To this end, we created a mutant construct of GLAST, in which the last amino acid in the GLAST-CT was changed from a methionine to an alanine (M542A). Immunoprecipitation experiments subsequently revealed that mutant GLAST-M542A was unable to bind to NHERF-1 or NHERF-2 (Fig. 2.3B). Together, these findings validate the results from the screens of the PDZ proteomic array and establish that both NHERF-1 and NHERF-2 can associate with GLAST in cells via interaction with the GLAST PDZ-binding motif.

We next examined the effects of the GLAST/NHERF interactions on GLAST function in cultured astrocytes. Western blot studies revealed that our cultured astrocytes were highly enriched in NHERF-2 relative to total brain tissue, but in contrast levels of NHERF-1 were much

lower than total brain tissue (Fig. 2.4A). We then examined which glutamate transporter, GLAST or GLT-1, was primarily responsible for the uptake of extracellular aspartate by these cells. Treatment of cultured astrocytes with TBOA (100μM), a non-selective glutamate transporter inhibitor, resulted in nearly complete inhibition of [^{3}H]-aspartate transport, while treatment of astrocytes with DHK (1mM), a GLT-1 selective blocker, only modestly prevented [^{3}H]-aspartate transport (Fig. 2.4B). These studies revealed that GLAST was the predominant functional glutamate transporter in our cultured astrocytes.

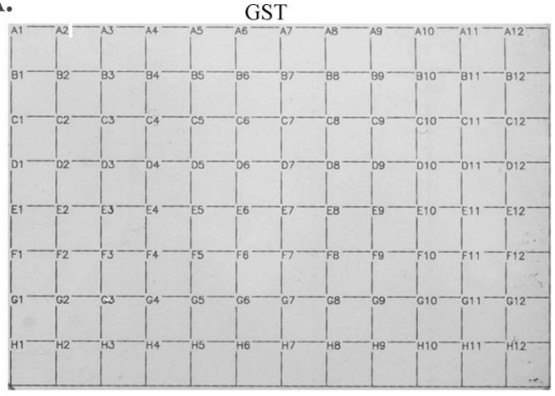
Given the high expression of GLAST and NHERF-2 in our cultured astrocytes, we then examined if knockdown of NHERF-2 in the cultured astrocytes might alter GLAST function. Treatment with siRNA resulted in a >90% knockdown of NHERF-2 in the cultured astrocytes (Fig. 2.5A). Strikingly, this reduction in NHERF-2 expression resulted in approximately a 50% decrease in the maximum total uptake of [^{3}H] aspartate by the NHERF-2 siRNA-treated astrocytes relative to control siRNA-treated astrocytes (Fig. 2.5B; paired t-test, $p < 0.001$).

Since the functional effects of NHERF-2 knockdown in cultured astrocytes were on the maximal activity of GLAST (not the apparent affinity for substrate), we assessed GLAST expression levels in the absence and presence of NHERF-2 interactions. Western blots revealed that siRNA knockdown of NHERF-2 in cultured astrocytes resulted in significantly reduced expression of GLAST (Fig. 2.5C). Pulse-chase metabolic labeling studies revealed that the half-life of the GLAST protein was reduced by 46% in NHERF-2 siRNA-treated astrocytes relative to control siRNA-treated astrocytes (Fig. 2.5D). Taken together, these studies indicate that NHERF-2 interactions with GLAST are important for GLAST stability and expression.

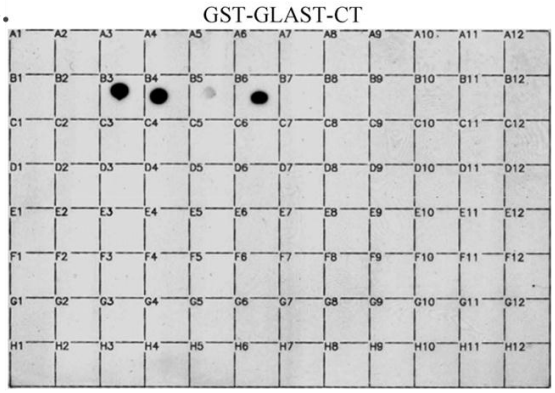
It has previously been shown that mGluR5 interacts with both NHERF-1 and NHERF-2 (Paquet et al., 2006a). Given that mGluR5 is known to be expressed in astrocytes (Mudo et al., 2007; Schools and Kimelberg, 1999; van den Pol et al., 1995) and to regulate GLAST expression

(Gegelashvili et al., 2000), we examined whether mGluR5 and GLAST might associate in a cellular context and whether this association might be dependent on association with the NHERF proteins. Immunoprecipitation of FLAG-GLAST from transfected HEK-293 cells resulted in the co-immunoprecipitation of mGluR5, providing evidence that GLAST and mGluR5 can indeed be found in complexes in a cellular context (Figure 2.6). However, immunoprecipitation of mutant FLAG-GLAST-M542A resulted in comparable levels of co-immunoprecipitation of mGluR5 to that observed with wild type GLAST, indicating that GLAST and mGluR5 association is not dependent on interactions with the NHERF proteins (Figure 2.6).

A.



B.



C.

Bin	PDZ Domain	Bin	PDZ Domain	Bin	PDZ Domain
A1	MAGI-1PDZ1	C9	E6TP1	F5	MUPP1 PDZ7
A2	MAGI-1 PDZ2	C10	ERBIN	F6	MUPP1 PDZ8
A3	MAGI-1 PDZ3	C11	ZO-1 PDZ1	F7	MUPP1 PDZ9
A4	MAGI-1 PDZ 4+5	C12	ZO-1 PDZ2	F8	MUPP1 PDZ10
A5	MAGI-2 PDZ1	D1	ZO-1 PDZ3	F9	MUPP1 PDZ12
A6	MAGI-2 PDZ2	D2	ZO-2 PDZ1	F10	PTPN13 PDZ1
A7	MAGI-2 PDZ3	D3	ZO-2 PDZ2	F11	PTPN13 PDZ2
A8	MAGI-2 PDZ4	D4	ZO-2 PDZ3	F12	PTPN13 PDZ3
A9	MAGI-2 PDZ5	D5	ZO-3 PDZ1	G1	PTPN13 PDZ4+5
A10	MAGI-3 PDZ1	D6	ZO-3 PDZ2	G2	PDZK1 PDZ1
A11	MAGI-3 PDZ2	D7	ZO-3 PDZ3	G3	PDZK1 PDZ2
A12	MAGI-3 PDZ3	D8	C2PA	G4	PDZK1 PDZ3
B1	MAGI-3 PDZ4	D9	GIPC	G5	PDZK1 PDZ4
B2	MAGI-3 PDZ5	D10	MALS-1	G6	PDZK2 PDZ1
B3	NHERF-1 PDZ1	D11	MALS-2	G7	PDZK2 PDZ2
B4	NHERF-1 PDZ2	D12	MALS-3	G8	PDZK2 PDZ3
B5	NHERF-2 PDZ1	E1	Densin-180	G9	PDZK2 PDZ4
B6	NHERF-2 PDZ2	E2	Rhophillin PDZ1	G10	LNX1 PDZ1
B7	PSD-95 PDZ1+2	E3	Rhophillin PDZ2	G11	LNX1 PDZ2
B8	PSD-95 PDZ3	E4	Harmonin PDZ1	G12	LNX1 PDZ3
B9	CNRasGEF	E5	Harmonin PDZ2	H1	LNX1 PDZ4
B10	CAL	E6	Neurabin	H2	LNX2 PDZ1
B11	nNOS	E7	Spinophillin	H3	LNX2 PDZ2
B12	INADL PDZ5	E8	α1-syntrophin	H4	LNX2 PDZ3
C1	INADL PDZ6	E9	β1-syntrophin	H5	LNX2 PDZ4
C2	INADL PDZ7	E10	β2-syntrophin	H6	LARG
C3	SAP97 PDZ1+2	E11	γ1-syntrophin	H7	MAST-2005
C4	SAP97 PDZ3	E12	γ2-syntrophin	H8	RA-GEF
C5	SAP102 PDZ1+2	F1	PAPIN PDZ1	H9	PDZ Rho-GEF
C6	SAP102 PDZ3	F2	MUPP1 PDZ1	H10	Tamalin
C7	Chap110 PDZ1+2	F3	MUPP1 PDZ4	H11	Shank1
C8	Chap110 PDZ3	F4	MUPP1 PDZ6	H12	Enigma

Figure 2.1. The C-terminus of GLAST binds selectively to NHERF PDZ domains. Control GST **(A)** or a GST-fusion protein comprising the last 25 amino acids of GLAST **(B)** were overlaid at 100 nM onto a proteomic array containing 96 distinct PDZ domains. In contrast to GST alone, the C-terminus of GLAST specifically bound to bins B3-B6, corresponding to the PDZ domains of NHERF-1 and NHERF-2. A complete list of the PDZ proteins on this array is shown in **(C)** and the "hits" from GST-GLAST-CT overlay are shown in red. The data shown here are representative of three independent experiments.

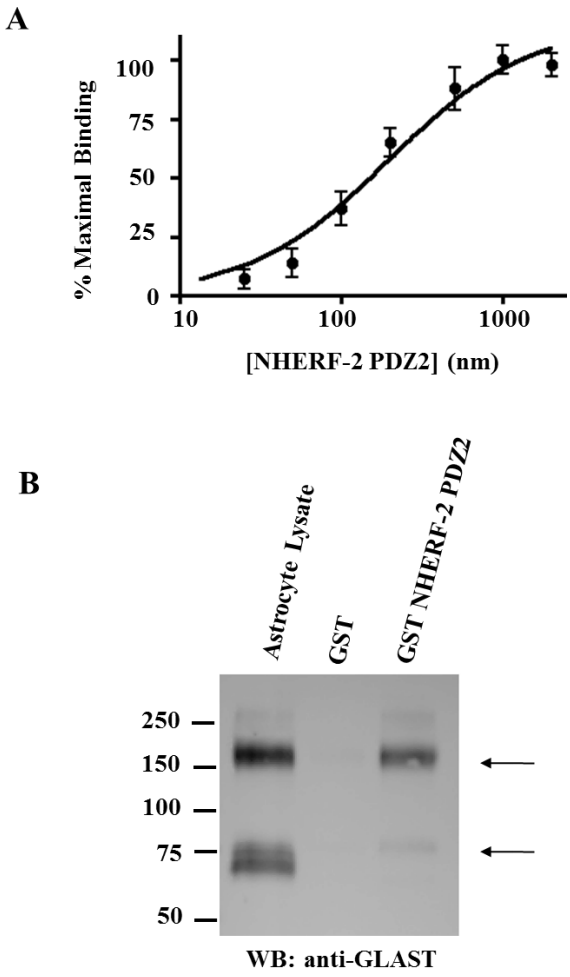


Figure 2.2. ***In vitro*** **association between GLAST and PDZ2 of NHERF-2. A)** Purified GST-GLAST-CT was transferred to nitrocellulose and overlaid with increasing concentrations of purified His/S-tagged NHERF-2-PDZ2. Bound NHERF-2 was visualized using S-protein HRP and quantified via densitometry. Saturation binding curves were generated, estimating the K_D for the NHERF-2/GLAST interaction at 196 nM. **B)** Full-length endogenous GLAST from astrocyte lysates was pulled down with purified GST-NHERF-2 (lane 3; arrows indicate GLAST monomers and oligomers) but not GST alone (lane 2).

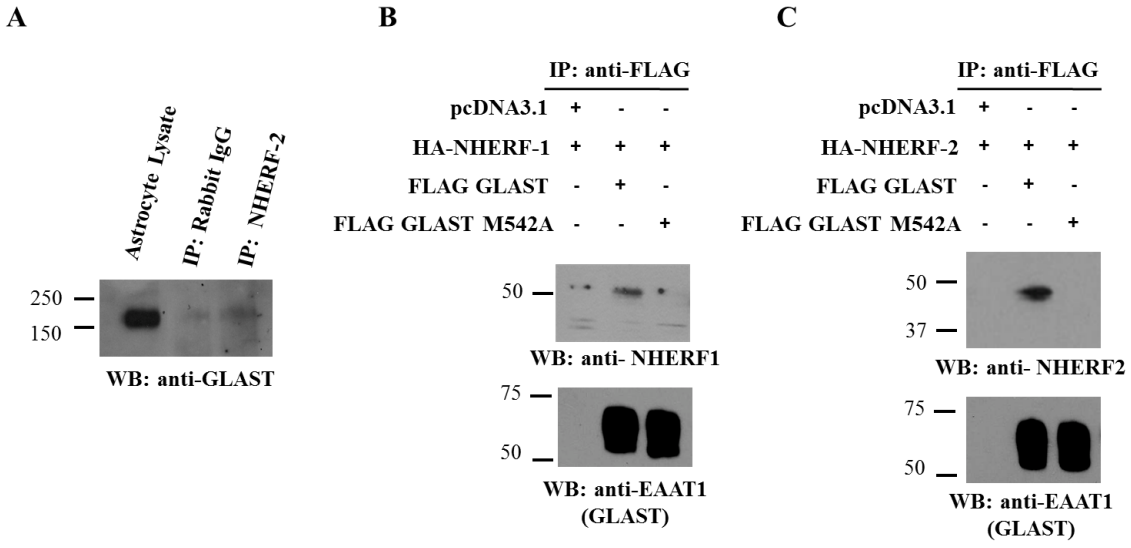


Figure 2.3. Association of NHERF-2 with GLAST in primary astrocytes and transfected cells. A) Immunoprecipitation of endogenous NHERF-2 from cultured primary rat astrocyte lysates resulted in the specific co-immunoprecipitation of GLAST, as detected with guinea pig anti-GLAST antibody (lane 2). The white band in lane 1 presumably is a blowout signal due to protein overloading. As a negative control, no GLAST co-immunoprecipitation was observed with an irrelevant rabbit antibody bound to A/G beads (lane 3). **B and C)** Mutation of the last amino acid in the GLAST-CT (methionine 542 to an alanine) disrupted cellular association of GLAST with NHERF-1 **(B)** or NHERF-2 **(C)** in HEK-293 cells transfected with HA-NHERF-1 or HA-NHERF-2 and either wild-type FLAG-GLAST or FLAG-GLAST-M542A mutant. The amount of co-immunoprecipitated NHERF-2 is shown in the top panel, while equal amounts of immunoprecipitated FLAG-GLAST and FLAG-GLAST-M542A, detected with rabbit anti-GLAST antibody, are shown in the lower panel (oligomer bands only shown). These data are representative of two to five independent experiments.

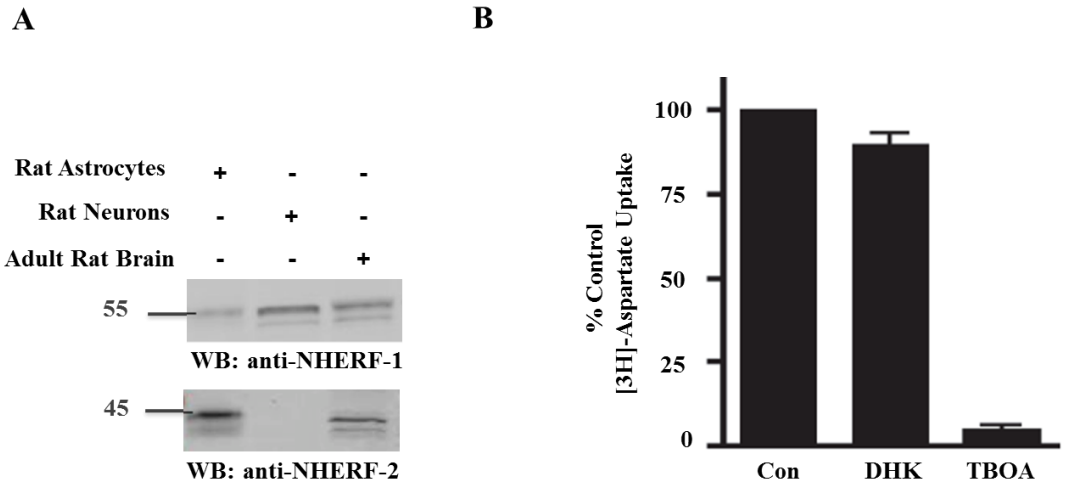


Figure 2.4. Cultured astrocytes are enriched in NHERF-2 and express functional GLAST. **A)** Equal protein levels of lysates from cultured rat astrocytes, cultured rat neurons, and total adult rat brain were subjected to gel electrophoresis. Western blot analysis revealed the cultured astrocytes were enriched in NHERF-2, relative to expression in brain lysates (lane 1 versus lane 3), while cultured neurons preferentially expressed NHERF-1, relative to total brain expression (lane 2 versus lane 3). These data are representative of four independent experiments. **B)** The uptake of [^{3}H]-aspartate was measured in cultured astrocytes in the absence and presence of the non-selective glutamate transporter inhibitor TBOA (100μM) and the GLT-1-selective blocker DHK (1mM). TBOA nearly completely inhibited [^{3}H]-aspartate transport, while DHK had only a modest effect (<10% decrease), suggesting that GLT-1 accounts for only a small percentage of the glutamate transport activity in these cultured astrocytes. The data shown here are means ± SEM for four experiments.

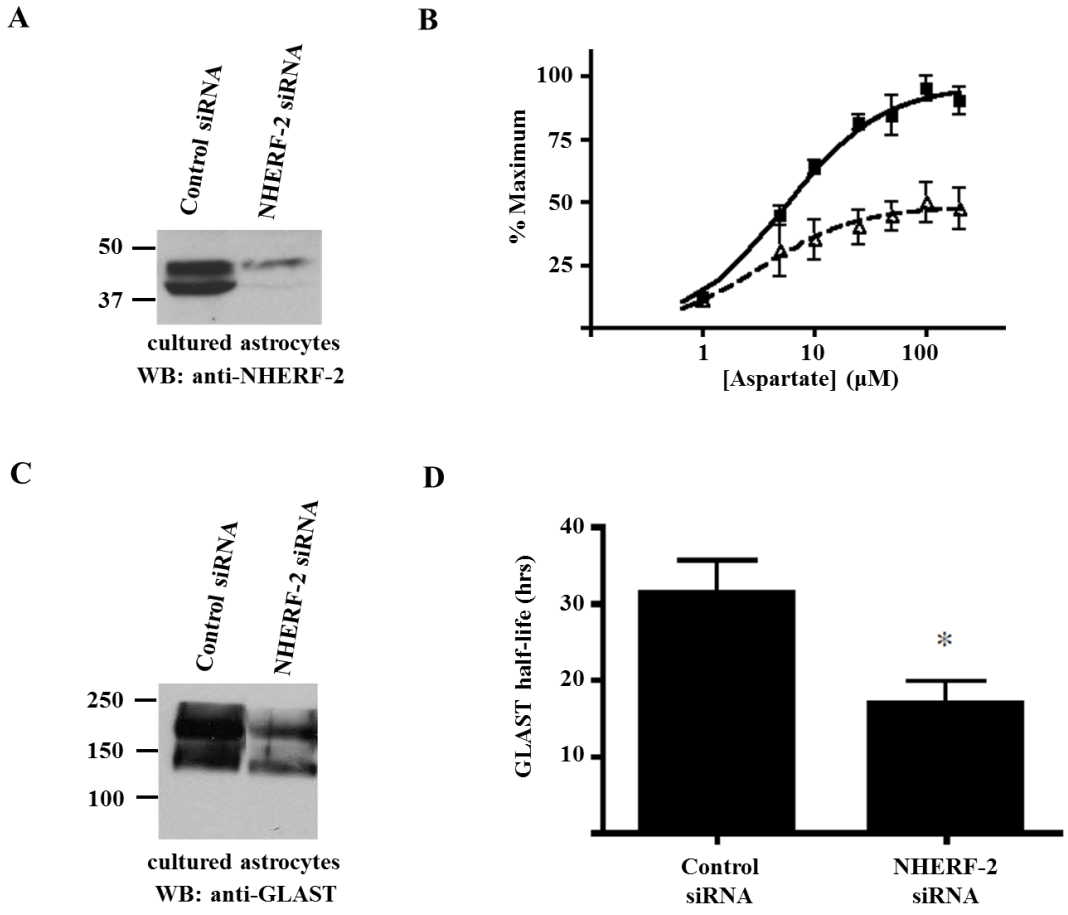


Figure 2.5. Knockdown of endogenous NHERF-2 in astrocytes reduces GLAST activity and protein levels. **A)** Treatment of astrocyte cultures with NHERF-2 siRNA (lane 2) versus scrambled control siRNA (lane 1) resulted in the specific knockdown of endogenous NHERF-2. **B)** GLAST activity was defined in cultured astrocytes by measuring uptake of different concentrations of [^{3}H]-aspartate in the presence of the GLT-1 inhibitor DHK (1mM). Data were normalized within each experiment and expressed as a percentage of the maximal uptake observed for either condition. Cultured astrocytes treated with NHERF-2 siRNA (open triangles, dashed line) transported nearly 50% less maximal [^{3}H] aspartate in comparison to control siRNA-treated astrocytes (black squares, solid line) containing normal endogenous levels of NHERF-2 (paired t-test, $p < 0.001$). The points and error bars represent means ± SEM for four independent

experiments. **C)** Cultured astrocytes were treated with control or NHERF-2 siRNA and subsequently harvested to check for GLAST expression. Western blot analysis revealed GLAST expression was reduced in cultured astrocytes treated with siRNA for NHERF-2, in comparison to control siRNA-treated astrocytes. **D)** Pulse-chase metabolic labeling studies revealed that knockdown of NHERF-2 in cultured primary astrocytes resulted in decreased half-life of the GLAST protein. The bars and error bars represent means and standard errors for three independent experiments (*, $p < 0.05$).

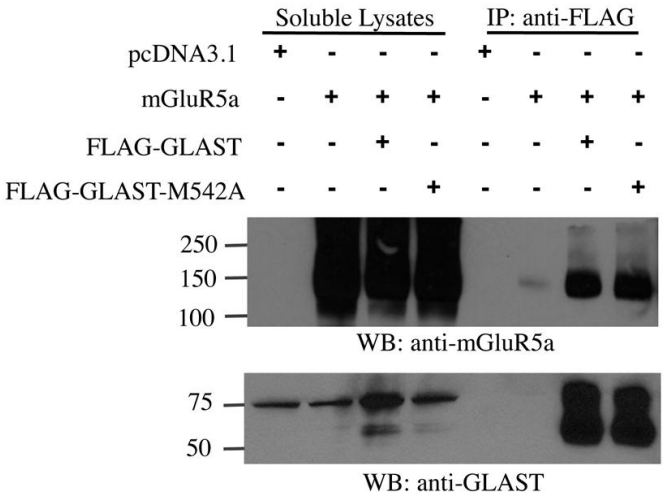


Figure 2.6. Metabotropic glutamate receptor 5 associates with GLAST in a NHERF-independent manner. HEK-293 cells overexpressing rat mGluR5a and either FLAG-GLAST or mutant FLAG-GLAST-M542A were solubilized and the receptors were immunoprecipitated with anti-FLAG antibodies. Robust co-immunoprecipitation of mGluR5a was observed with both wild-type and mutant GLAST, revealing the presence of cellular complexes of GLAST and mGluR5a that were not dependent upon associations with NHERF proteins. These studies are representative of three independent experiments.

2.4. Discussion

The studies described here have identified NHERF-2 as a novel interacting partner for the astrocytic glutamate transporter GLAST. Previously, the related protein NHERF-1 was identified as a GLAST binding partner by Lee *et al*.(Lee et al., 2007a). Our findings expand on this earlier report in several important ways. First, Lee *et al*. examined only the specific interaction of GLAST and NHERF-1, but did not screen for potential GLAST interactions with other PDZ scaffolds. In our studies, we screened the GLAST-CT for potential binding to 96 distinct PDZ domains and established a high specificity of binding to the PDZ domains from NHERF-1 and NHERF-2. Secondly, Lee *et al*. discounted the possibility that GLAST might interact with NHERF-2 on the basis of light microscopy studies showing a high concentration of NHERF-2 labeling around CNS blood vessels, which they interpreted to indicate NHERF-2 expression in endothelial cells but not neurons or astrocytes (Lee et al., 2007a). However, in electron microscopy studies that were published contemporaneously with the studies by Lee *et al*., we showed that NHERF-2 expression in the brain is actually highest in astrocytes (Paquet et al., 2006b). The perivascular astrocytes surrounding blood vessels express particularly high levels of NHERF-2 (Paquet et al., 2006b), which suggests an explanation for the observations made at the light microscopy level by Lee *et al.* concerning intense NHERF-2 labeling in the vicinity of blood vessels. The question of whether GLAST associates with NHERF-1, NHERF-2 or both NHERF proteins in brain astrocytes is of critical importance in planning future studies focused on assessing the *in vivo* significance of GLAST/PDZ interactions. Since there is substantial functional redundancy between the NHERF proteins (Weinman et al., 2006), our finding that GLAST interacts with both of the NHERF proteins reveals that it will be important to study double NHERF-1/NHERF-2 knockout mice in order to fully assess the effects of PDZ scaffold regulation of GLAST function *in vivo*.

A third way that our studies expand on the earlier findings by Lee *et al*. is in providing evidence that GLAST/NHERF interactions have effects on GLAST functional activity in astrocytes. The work by Lee *et al*. solidly established the GLAST/NHERF-1 interaction (Lee et al., 2007a), and a follow-up study revealed that knockdown of NHERF-1 expression in COS-7 cells resulted in reduced activity of transfected GLAST (Sullivan et al., 2007), but this work did not address the physiological importance of endogenous GLAST/NHERF associations in astrocytes. Our studies reported here demonstrate that disruption of the GLAST/NHERF-2 interaction in primary astrocytes results in decreased GLAST activity due to impaired stability of the GLAST polypeptide. These findings are analogous to the effects of PDZ scaffold interactions with certain other neurotransmitter transporters. For example, the associations of EAAT4 with GTRAP48 (Jackson et al., 2001), the dopamine transporter DAT with PICK1 (Torres et al., 2001), and the GLT-1 splice variant GLT-1b with PSD-95 (Gonzalez-Gonzalez et al., 2008) result in enhanced surface expression and activity of the transporters. The detailed molecular mechanism(s) by which PDZ scaffold interactions can enhance transporter stability and function are not presently understood, but will be an interesting area for future investigation.

Since the metabotropic glutamate receptor mGluR5, like GLAST, is expressed in astrocytes (Mudo et al., 2007; Schools and Kimelberg, 1999; van den Pol et al., 1995) and associates with NHERF proteins (Paquet et al., 2006a), we wondered if mGluR5 and GLAST might associate in cells in a NHERF-mediated manner. Our co-immunoprecipitation studies revealed that mGluR5 and GLAST can indeed be found in cellular complexes together, but that these associations do not seem to be dependent on the joint interactions of mGluR5 and GLAST with NHERF proteins. It is not clear whether mGluR5 and GLAST directly interact with each other or if instead they are components of large multi-protein complexes that might also involve (but not be dependent upon) NHERF-1 and/or NHERF-2. There is precedent for the idea that receptors and transporters for the same neurotransmitter can associate with each other. For

example, it has been shown that dopamine D2 receptors and the dopamine transporter DAT form complexes in cells (Lee et al., 2007b). Since there is extensive functional evidence for cross-talk between metabotropic glutamate receptors and glutamate transporters (Gegelashvili et al., 2000; Huang et al., 2004; Vermeiren et al., 2005), it may be of significant future interest to determine whether the formation of cellular complexes between the receptors and transporters plays a role in facilitating such cross-talk.

In summary, we have found a novel interaction between GLAST and NHERF-2 that modulates GLAST functional activity in astrocytes. GLAST and other glutamate transporters are considered to be prime targets for the development of future therapeutics aimed at reducing neurodegeneration in stroke and other pathological conditions (Beart and O'Shea, 2007; Rothstein et al., 2005). Thus, scaffolds that modulate glutamate transporter function may also be excellent targets for new therapeutics. Given the short peptide motifs with which PDZ domains interact, these domains are attractive candidates for therapeutic intervention (Dev, 2004), and in fact small peptidomimetic molecules that bind to and specifically regulate the NHERF PDZ domains are already being developed (Mayasundari et al., 2008). Thus, future work on GLAST/NHERF interactions may both shed light on the mechanisms controlling GLAST function *in vivo* and also provide opportunities for the development of novel therapeutics aimed at modulation of GLAST function for the treatment of neurological disorders.

2.5. Materials and Methods

Overlay of PDZ Array: Fusion proteins were purified and overlays of the PDZ domain array were performed as previously described (Fam et al., 2005; He et al., 2006). Briefly, 1 μg of His- and S-tagged PDZ domain fusion proteins were spotted onto nitrocellulose, dried overnight, and then overlaid with GST-alone (control) or GST-GLAST-CT. Membranes were washed and incubated

with an HRP-coupled anti-GST monoclonal antibody (Amersham Pharmacia Biotech) and binding of GLAST-CT fusion protein was visualized using enhanced chemiluminescence.

Cell Culture and Transfection: HEK-293T (ATCC) cells were cultured in Dulbecco's modified Eagle medium containing GlutaMAX™, supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin, and were maintained at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Transfections were performed with Lipofectamine 2000, as previously described(Lau and Hall, 2001). DNA constructs used were rat GLAST in pcDNA3 (kindly provided by Jeff Rothstein, Johns Hopkins), human FLAG-GLAST (kindly provided by Susan Amara, Univ. Pittsburgh), rat mGluR5a in pRK5 (kindly provided by Jean-Philippe Pin, Institut de Genomique Fonctionnelle), rabbit HA-NHERF-1 in pBK-CMV, and human HA-NHERF-2 in pBK-CMV.

Preparation of Primary Astrocytes: Purified primary astrocyte cultures were prepared by the method of McCarthy and de Vellis (McCarthy and de Vellis, 1980). All animal work was performed under the guidance of the National Institute of Health Guide for the Care and Use of Laboratory Animals. In brief, neocortices were dissected from E18-19 Sprague Dawley rat embryos and dissociated in medium by trituration. The cells were re-suspended in GlutaMAX™ DMEM supplemented with 10% FBS and 1% PenStrep in poly-D-lysine coated tissue culture flasks. The medium was changed the next day and then subsequently every 48 hours. After 6-8 days, cells were shaken overnight (280-310 rpm) to remove microglia and oligodendrocytes and passaged 48 hours later onto poly-D-lysine coated 100 mm culture dishes for future experimental use. Preparation of primary cortical neuron cultures was performed as described (Calin-Jageman et al., 2007).

Fusion Protein Overlays: Overlay assays, immunoprecipitation experiments, and Western blotting were performed as previously described (Lau and Hall, 2001). For overlay experiments, 1 μg of

purified protein was separated by SDS-PAGE gel electrophoresis, transferred onto nitrocellulose, and overlaid with purified His/S-tagged NHERF-2 (PDZ domain 2) fusion protein (prepared using a pET30A construct) in increasing concentrations. Binding of His/S-tagged NHERF-2 to GLAST was visualized with S-protein HRP and quantified via densitometry. Numbers were expressed as a percentage of maximum binding in order to generate a binding curve to estimate the affinity of the GLAST and NHERF-2 (PDZ2) interaction.

Immunoprecipitation: Immunoprecipitation experiments with transfected cell lysates were performed using anti-FLAG M2 affinity gel (Sigma), whereas immunoprecipitation experiments with cultured astrocyte lysates were performed with NHERF-2 antibody bound to Protein A/G agarose beads (Pierce). Astrocyte lysates were also incubated with purified NHERF-2 or GST-alone bound to beads for GLAST pull-downs. To detect endogenous and/or recombinant NHERF-1, NHERF-2, and GLAST, the reagents used were S-protein HRP conjugate (1:4,000, Novagen), polyclonal guinea pig anti-GLAST (1:10,000, Chemicon), rabbit anti-EAAT1 (H-50, Santa Cruz), rabbit anti-NHERF-1 (1:7,000) and rabbit anti-NHERF-2 (1:5000) (Yun et al., 1998).

Site-Directed Mutagenesis and siRNA Treatments: Mutagenesis of the last amino acid of the GLAST C-terminus from a methionine to an alanine was performed using the QuikChange Site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. Primers used for PCR were 5'-GACAGCGAAACCAAGGCGTAG-3' and its reverse complement. Sequencing was performed to confirm mutation of only the target bases. Knockdown of NHERF-2 was accomplished using TransIT-siQuest transfection reagent (Mirus) following the manufacturer's instructions. NHERF-2 siRNA was Silencer pre-designed siRNA (ID# 55838; Ambion). Control siRNA was Silencer Negative Control #1 siRNA (Ambion).

Amino Acid Uptake Assays: For the amino acid uptake experiments, astrocytes were incubated at 37°C in assay buffer containing 1 mM of the GLT-1-selective inhibitor dihydrokainate (DHK) (Tocris), followed by increasing concentrations of [^{3}H]aspartate for 6 minutes in a 37°C water bath. Uptake was stopped with three washes of ice-cold uptake buffer containing choline chloride in replacement of sodium chloride. Cells were lysed with 0.5 M NaOH and samples were counted in 30% Scintisafe (Fisher).

Metabolic Labeling Studies: Pulse-chase assays were performed as previously described (Balasubramanian et al., 2007). Briefly, primary astrocytes that had been treated with either control siRNA or NHERF-2 siRNA were incubated in methionine-free DMEM for thirty minutes, then 60μCi of Redivue L-[^{35}S]-methionine (Amersham Biosciences) was added to each plate to incubate at 37°C for an additional 30 minutes. Cells were washed and immediately chased with 3 mM cold L-methionine for various time points. Samples were adjusted to normalize for protein concentration, GLAST was immunoprecipitated using anti-GLAST antibodies, and the amount of ^{35}S incorporated into the GLAST protein was determined at the different time points.

2.6. Acknowledgements

I would like to thank Drs. Maryse Paquet, Matthew Assay, and Kevin Paavola for their assistance with the GLAST and PDZ array studies, GLAST expression and pulse-chase metabolic labeling studies in astrocytes, and GLAST and NHERF PDZ domain overlays. I would like to thank Rachel Reiff for assistance with the mGluR5 and GLAST immunoprecipitation studies.

CHAPTER 3

Group II Metabotropic Glutamate Receptors Interact Distinctly with the NHERF Scaffolding Proteins

[4] Chapter contains select material from the manuscript in preparation on Group II mGluRs and NHERFs (in preparation).

3.1. Summary

In order to discern novel interacting partners of Group II mGluRs and potentially shed light on innovative therapeutic strategies targeting these receptors, we screened the C-termini of mGluR2 and mGluR3 for interactions with PDZ domains. These screens revealed that the Na+/H+ Exchanger Regulatory Factor 1 or 2 (NHERF-1, NHERF-2) proteins are candidate interacting partners. Further work demonstrated that both mGluR2 and mGluR3 can associate with NHERF-1 and NHERF-2 in cells, as assessed via co-immunoprecipitation studies, although a higher fraction of cellular mGluR3 associates with the NHERFs, relative to mGluR2. Site-directed mutagenesis studies subsequently revealed that this interaction is dependent on the hydrophobicity of the last amino acid of mGluR2 and mGluR3, as mutagenesis from a leucine to an alanine was sufficient to disrupt NHERF association. Functional studies revealed that disruption of PDZ interactions with mGluR2 enhanced receptor signaling to Akt. For the weaker-signaling mGluR3, in contrast, no significant effect of PDZ associations could be established for receptor signaling. Studies of mGluR2 or mGluR3 signaling in astrocytes in which NHERF expression was reduced by gene knockout and siRNA knockdown techniques revealed that the observed differences in signaling between WT and mutant mGluR2 were likely not due to disruption of interactions with the NHERF proteins.

3.2. Introduction

Metabotropic glutamate receptors have emerged as intriguing therapeutic targets towards the treatment of neurodegenerative and neuropsychiatric disorders. For example, the success of a Stage II clinical trial of a systemically-active Group II mGluR agonist has ushered in a resurgence in studying Group II mGluRs as neuropsychiatric targets, as treatment with a Group II mGluR agonist alleviated the positive and negative symptoms associated with schizophrenia similar to traditional antipsychotics, without the accompanying side-effects of weight gain, extrapyramidal

motor symptoms, and elevation of prolactin (Patil et al., 2007). Moreover, recent reviews have highlighted the roles of these receptors in anxiety, drug addiction, and schizophrenia (Nicoletti et al., 2011). Despite this increased interest in these receptors, many questions remain unanswered. For example, most of the reagents available to study Group II mGluRs cannot differentiate between the two receptors. Besides the controversial mGluR3-selective agonist NAAG (Neale et al., 2011), no other sub-type selective mGluR3 agonist exists. In contrast, positive allosteric modulators, or PAMs, have been developed for mGluR2 (Johnson et al., 2003; Sheffler et al., 2011). In light of these challenges in distinguishing the receptors, a variety of approaches are often taken to discern the downstream functions of activation of Group II mGluRs including a combination of genetic approaches in which mGluR2, mGluR3, or both mGluR2 and mGluR3 are deficient, the culturing of particular CNS cell types that express one or both receptors, and the application of currently available drugs to selectively activate receptor populations.

Another approach in elucidating the function of Group II mGluRs is to identify new interacting partners, which may present an alternative site of receptor modulation that is distinct from the orthosteric binding site. A handful of Group II mGluR-interacting partners have been identified including tamalin (Kitano et al., 2002), protein kinase A or PKA (Cai et al., 2001; Schaffhauser et al., 2000), GRKs (Iacovelli et al., 2009), beta-arrestins (Iacovelli et al., 2009), RanBPM (Seebahn et al., 2008), PICK1 (Hirbec et al., 2002), GRIP (Hirbec et al., 2002) and protein phosphatase 2C or PP2C (Flajolet et al., 2003). However it is unknown what the function and therefore significance may be for most of these interactions, save for the association between Group II mGluRs and the arrestins (Iacovelli et al., 2009). Both mGluR2 and mGluR3 contain a putative PDZ-interacting motif on their C-termini, which may provide an important regulatory site for Group II mGluR activity. Indeed, some of the candidate Group II mGluR-interacting partners previously identified are PDZ proteins. Thus, we endeavored to use our unique PDZ

proteomic array of 96 distinct PDZ proteins to discover novel Group II mGluR interacting partners that might modulate receptor function and therefore provide new therapeutic targets.

3.3. Results

A proteomic array of 96 distinct type I PDZ domains was screened with fusion proteins comprising the last 25 amino acids of the C-termini (CT) of mGluR2 or mGluR3. The two fusion proteins bound to a distinct set of PDZ proteins (Figure 3.1). The mGluR2- and mGluR3-CT bound robustly to PDZ domains from the the Na+/H+ Exchanger Regulatory Factors 1 and 2 (NHERF-1, NHERF-2). In particular, mGluR2-CT robustly bound to PDZ domain 1 and PDZ domain 2 of NHERF-1 and NHERF-2, while mGluR3-CT bound robustly to both PDZ domains of NHERF-2, yet appeared to preferentially bind PDZ domain 1 of NHERF-1. Of noteworthy interest, mGluR3-CT, as well as to a lesser extent mGluR2-CT, also bound to the cystic fibrosis transmembrane conductance regulator-associated ligand (CAL; also known as PIST, GOPC, and FIG). Importantly, only a handful of additional weak interactions were detected, increasing confidence in the specificity of the observed associations.

After comparing the NHERF proteins and CAL as candidate Group II-mGluR interacting partners, we decided to focus on the NHERF proteins, which associated with both mGluR2 and mGluR3, whereas CAL interacted only with mGluR3. Additionally, NHERF-2 has previously been shown to be an interacting partner for mGluR5 (Paquet et al., 2006a), and as shown in Chapter 2, both NHERF-1 and NHERF-2 have also been shown to associate with the astrocytic glutamate transporter GLAST (Chapter 2). Therefore, we endeavored to explore how interaction with NHERF-1 and/or NHERF-2 might regulate Group II mGluR function.

As initial identification of the Group II mGluR and NHERF interaction relied on the Group II mGluR C-termini, it was next necessary to determine whether full-length mGluR2 and mGluR3 might associate with the NHERF proteins in cells. To this end, co-immunoprecipitation

experiments were performed using human embryonic kidney (HEK293T) cells that were transiently transfected with cDNAs encoding pcDNA3.1 (mock vector), rat mGluR2, rat mGluR3, FLAG-tagged NHERF-1 or FLAG-tagged NHERF-2. Robust immunoprecipitation of FLAG-tagged NHERF-1 or NHERF-2 resulted in the co-precipitation of either mGluR2 or mGluR3 (Figure 3.2). Interestingly, a higher fraction of cellular mGluR3 consistently associated with both NHERF-1 and NHERF-2, relative to mGluR2, highlighting a potential difference between these two receptors and their ability to interact with the NHERF proteins. Since enhanced recruitment of the NHERF proteins to receptors following agonist stimulation has been previously documented (Hall et al., 1998b; Sitaraman et al., 2002; Takahashi et al., 2006), we examined if treatment of HEK293T cells expressing mGluR2 with 1 μM LY 354740, a Group II mGluR agonist, for 30 minutes prior to co-immunoprecipitation, might enhance its association with the NHERF proteins; however, no difference was observed (data not shown).

Interaction of type 1 PDZ proteins with their cellular partners has been shown to usually be dependent on the last few amino acids of the interacting partners (Doyle et al., 1996; Niethammer et al., 1998). To this end, the last amino acid of both the mGluR2 and the mGluR3 CT was mutated from a leucine to an alanine (L872A and L879A respectively). It was then examined if these mutant constructs could associate with the NHERF proteins via co-immunoprecipitation. Both mGluR2 L872A and mGluR3 L879A bound poorly to either NHERF protein (Figure 3.3, NHERF-1 data not shown), thereby demonstrating the requirement of this last amino acid for NHERF interaction.

After characterizing the interaction, we next decided to study how Group II mGluR interaction with the NHERF proteins might regulate downstream signaling. Studies of Group II mGluR signaling in HEK293T cells heterologously overexpressing either mGluR2 and mGluR3 did not result in any significant activation of phospo-ERK (pERK) or phospho-AKT (pAKT) at all time-points examined (5 min, 10 min, 30 min, 60 min), which was in contrast to dopamine-

mediated activation of pERK responses downstream of D_2R dopamine receptor activation or epidermal growth factor-mediated activation of pAKT responses downstream of EGF epidermal growth factor receptor activation (data not shown). Studying the downstream signaling of the Group II mGluRs has been noted to be particularly challenging in the field, with mGluR3 being the more challenging receptor to study, presumably because it has the highest affinity for glutamate (see Table 1.1) and it is near to impossible to remove glutamate from the cell culturing conditions (Wroblewska et al., 2011; Wroblewska et al., 1997). To this end, certain groups have co-expressed glutamate transporters, such as GLAST, along with either mGluR2 or mGluR3, to arguably control extracellular glutamate levels, analogous to the function of the glutamate transporters in the brain (Schoepp et al., 1997). Cultured astrocytes are known to express both NHERF-1 and NHERF-2 and have high levels of GLAST (Ritter et al., 2011), and reportedly express endogenous mGluR3 (Aronica et al., 2003; Bruno et al., 1998; Bruno et al., 1997; Ciccarelli et al., 2007; D'Onofrio et al., 2003; D'Onofrio et al., 2001; Durand et al., 2010). Given the challenges with studying mGluR3 in heterologous over-expression systems, we decided to employ cultured astrocytes as a model system to study mGluR2 and mGluR3 signaling. However, despite employing a variety of astrocyte culturing methods, including preparing cultures from mice versus rats, making primary versus secondary astrocyte cultures, adding exogenous growth factors such as epidermal growth factor (EGF, 100 ng/mL) or the B-27 supplement (see Appendix, Figure A.2), and modeling hypoxic conditions via the stabilization of HIF-1α, we were unable to detect expression of endogenous astrocytic mGluR3 under any condition tested. Furthermore, we did not observe any functional evidence that mGluR3 was endogenously present in our astrocyte cultures (see Appendix, Figure A.2). Thus, our conclusion was that Group II mGluR expression was being lost during the culturing process of our astrocyte cultures.

Given the lack of endogenous Group II mGluR expression in our primary astrocyte cultures, we decided to reintroduce Group II mGluRs back into these astrocyte cultures via transfection. Since we wanted to compare NHERF regulation of both mGluR2 and mGluR3, we also performed studies in which we separately expressed either mGluR2 or mGluR3. As mentioned above, there is extensive evidence that mGluR3 is widely expressed in astrocytes in many brain regions *in vivo* (Muly et al., 2007; Ohishi et al., 1994; Petralia et al., 1996; Sun et al., 2013; Tamaru et al., 2001), and expression of mGluR2 has been described in certain populations of astrocytes as well (Phillips et al., 2000). Since activation of Group II mGluRs has been shown to modulate both ERK and AKT, we examined these two signaling outputs in astrocyte cultures and found that both mGluR2 and mGluR3 could activate AKT, whereas no appreciable activation of pERK was observed following mGluR stimulation (see Appendix, A.3). Subsequent studies compared the abilities of mutant mGluR2 L872A and mGluR3 L879A to signal to AKT, as NHERF proteins have been shown to regulate AKT signaling that is downstream of receptor tyrosine kinases (Pan et al., 2008; Takahashi et al., 2006).

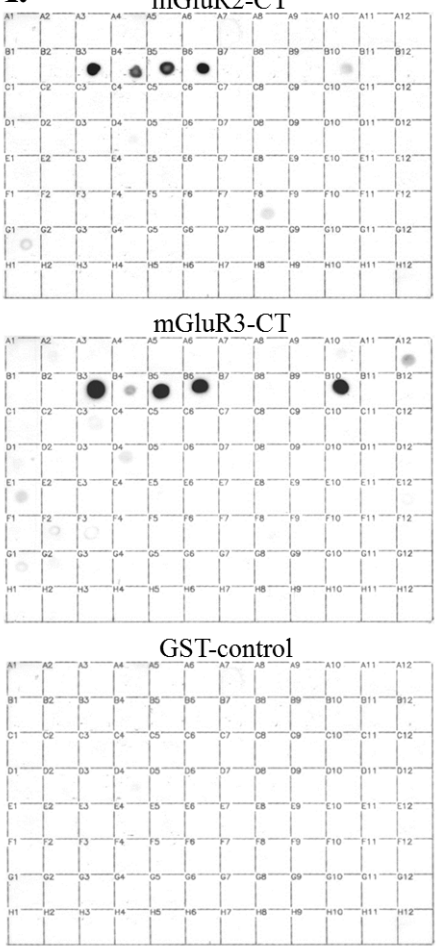
Examination of mGluR2 versus mGluR2 L872A signaling in the primary astrocytes revealed that mutant mGluR2 signaled more robustly to AKT, as assessed by increased ratios of phosphorylated Ser473 normalized to total AKT (Figure 3.4 A). This difference did not reflect a difference in the time course of AKT signaling, as there was no difference in the magnitude of the effect at different time points. In contrast, WT mGluR3 and mutant L879A signaled to AKT comparably, both with a much smaller fold increase in AKT phosphorylation than was observed for mGluR2 (Figure 3.4 B). These data provide evidence that disruption of PDZ domain interactions with the C-terminus of mGluR2 can negatively regulate downstream mGluR2-mediated AKT signaling.

In order to determine if interactions with the NHERF proteins, as opposed to other PDZ partners, were important for modulation of mGluR2 signaling to AKT, we compared responses in

astrocyte cultures prepared from WT, NHERF-1 KO, NHERF-2 KO, or NHERF-2 KO astrocytes treated with control siRNA or NHERF-1 siRNAs. However, no differences were observed when comparing mock, mGluR2, or mGluR3 responses across all culture types for Ser473-mediated AKT responses (Figure 3.5 A) or Thr308-mediated AKT responses (Figure 3.5 B). Additionally, it was confirmed that moderate knockdown of NHERF-1 was achieved (Figure 3.6 C) in the NHERF-2 KO cultures, thereby creating a condition in which little full length NHERF proteins would be expressed. These data suggest that loss of NHERF-1, NHERF-2, or both NHERF proteins does not affect Group II mGluR-mediated AKT responses.

Given that NHERF proteins are known to sometimes facilitate switches in G protein-coupling for their GPCR-interacting partners (Ritter and Hall, 2009), we examined the G protein-pathway(s) utilized by mGluR2 for signaling to AKT in astrocyte cultures prepared from WT, NHERF-1 KO, or NHERF-2 KO mice. Treatment of mGluR2-transfected astrocyte cultures with pertussis toxin, (100 ng/ml) a selective inhibitor of Gαi/o, abolished agonist-dependent increases in AKT phosphorylation at both sites measured (Figure 3.6), revealing that mGluR2-mediated increases in AKT are strictly dependent on Gαi/o across all culture conditions.

A.



B.

Bin	PDZ Domain	Bin	PDZ Domain	Bin	PDZ Domain
A1	MAGI-1 PDZ1	C9	ERBIN PDZ	F5	MUPP1 PDZ12
A2	MAGI-1 PDZ2	C10	ZO-1 PDZ1	F6	MUPP1 PDZ13
A3	MAGI-1 PDZ3	C11	ZO-1 PDZ2	F7	PTPN13 PDZ1
A4	MAGI-1 PDZ4+5	C12	ZO-1 PDZ3	F8	PTPN13 PDZ3
A5	MAGI-2 PDZ1	D1	ZO-2 PDZ1	F9	PTPN13 PDZ4+5
A6	MAGI-2 PDZ2	D2	ZO-2 PDZ2	F10	PDZK1 PDZ1
A7	MAGI-2 PDZ3	D3	ZO-2 PDZ3	F11	PDZK1 PDZ2
A8	MAGI-2 PDZ4	D4	ZO-3 PDZ1	F12	PDZK1 PDZ3
A9	MAGI-2 PDZ5	D5	ZO-3 PDZ2	G1	PDZK1 PDZ4
A10	MAGI-3 PDZ1	D6	ZO-3 PDZ3	G2	PDZK2 PDZ1
A11	MAGI-3 PDZ2	D7	C2PA PDZ	G3	PDZK2 PDZ2
A12	MAGI-3 PDZ3	D8	GIPC PDZ	G4	PDZK2 PDZ3
B1	MAGI-3 PDZ4	D9	MALS-1 PDZ	G5	PDZK2 PDZ4
B2	MAGI-3 PDZ5	D10	MALS-3 PDZ	G6	LNX1 PDZ1
B3	NHERF-1 PDZ1	D11	Densin-180 PDZ	G7	LNX1 PDZ2
B4	NHERF-1 PDZ2	D12	Rhophilin PDZ1	G8	LNX1 PDZ3
B5	NHERF-2 PDZ1	E1	Rhophilin PDZ2	G9	LNX1 PDZ4
B6	NHERF-2 PDZ2	E2	Harmonin PDZ1	G10	LNX2 PDZ1
B7	PSD-95 PDZ1+2	E3	Harmonin PDZ2	G11	LNX2 PDZ2
B8	PSD-95 PDZ3	E4	Neurabin PDZ	G12	LNX2 PDZ4
B9	PDZ-GEF-1 PDZ	E5	Spinophilin PDZ	H1	PTPN4 PDZ
B10	CAL PDZ	E6	α1 syntrophin PDZ	H2	RHO-GEF PDZ
B11	nNOS PDZ	E7	β1 syntrophin PDZ	H3	RA-GEF PDZ
B12	INADL PDZ5	E8	β2 syntrophin PDZ	H4	Enigma PDZ
C1	INADL PDZ6	E9	γ1 syntrophin PDZ	H5	LARG PDZ
C2	SAP97 PDZ1+2	E10	γ2 syntrophin PDZ	H6	MAST-205 PDZ
C3	SAP97 PDZ3	E11	PAPIN PDZ1	H7	PTPN3 PDZ
C4	SAP102 PDZ1+2	E12	MUPP1 PDZ1	H8	Shank1 PDZ
C5	SAP102 PDZ3	F1	MUPP1 PDZ6	H9	Tamalin PDZ
C6	Chapsyn110 PDZ1+2	F2	MUPP1 PDZ7	H10	PAR-3 PDZ1
C7	Chapsyn110 PDZ3	F3	MUPP1 PDZ8	H11	PAR-3 PDZ2
C8	E6TP1 PDZ	F4	MUPP1 PDZ10	H12	PAR-3 PDZ3

Red = Common, Green = m2-CT, Blue = m3-CT

Figure 3.1. The C-termini of mGluR2 and mGluR3 binds selectively to NHERF PDZ domains. **A)** The C-termini (CT) of mGluR2 and mGluR3 were screened on an array of 96 distinct PDZ domains. Both PDZ domains of NHERF-1 and NHERF-2 were the top hits for mGluR2 and mGluR3, while GST alone was negative. A complete list of the PDZ proteins on this array is shown in **(B).** The "hits" from GST-mGluR2/3-CT overlay are shown in red, the "hits" for GST-mGluR2-CT are shown in green, and the "hits" for mGluR3-Ct are shown in blue. The data shown here are representative of three independent experiments.

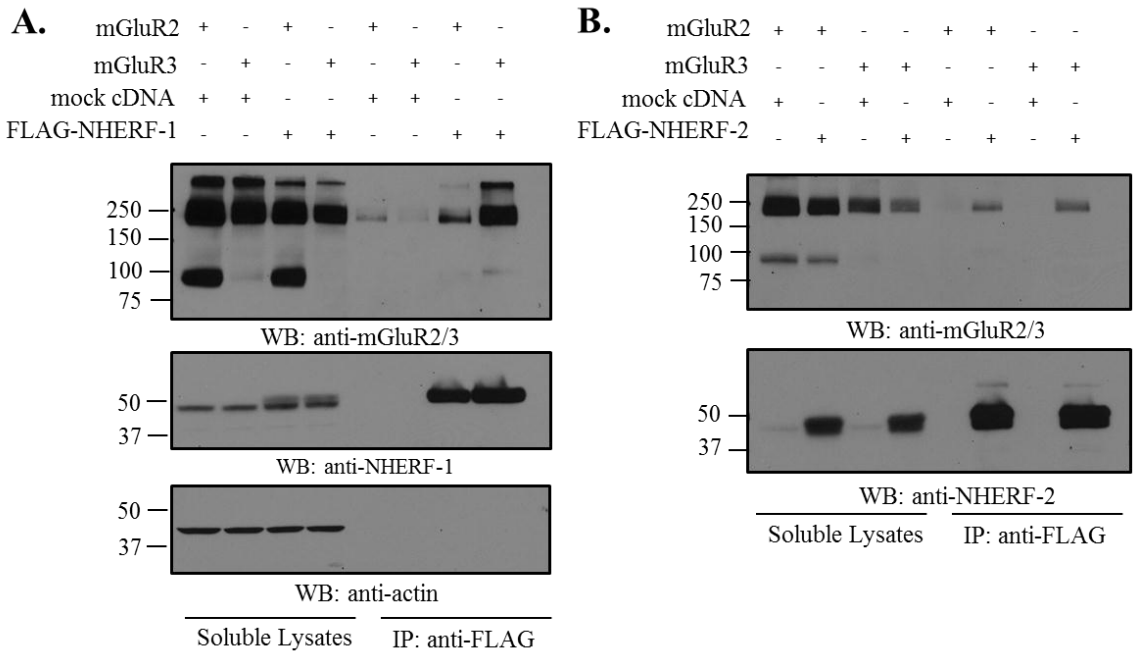


Figure 3.2. Group II mGluRs associate with NHERF-1 or NHERF-2 in a cellular context. HEK293T cells were transiently transfected with mock, mGluR2, mGluR3, or FLAG NHERF-1 **(A)** or FLAG-NHERF-2 **(B)** cDNAs. **A)** Robust immunoprecipitation of NHERF-1 with FLAG beads was achieved (middle panel; lanes 7 and 8), which coincided with the co-precipitation of either mGluR2 or mGluR3 (top panel; lanes 7 and 8) over background (top panel; lanes 5 and 6). Similar results were obtained in at least 2-3 separate experiments. **B)** Robust immunoprecipitation of NHERF-2 with FLAG beads was achieved (middle panel; lanes 6 and 8), which coincided with the co-precipitation of either mGluR2 or mGluR3 (top panel; lanes 6 and 8) over background (top panel; lanes 5 and 7). Similar results were obtained in at least 5 separate experiments.

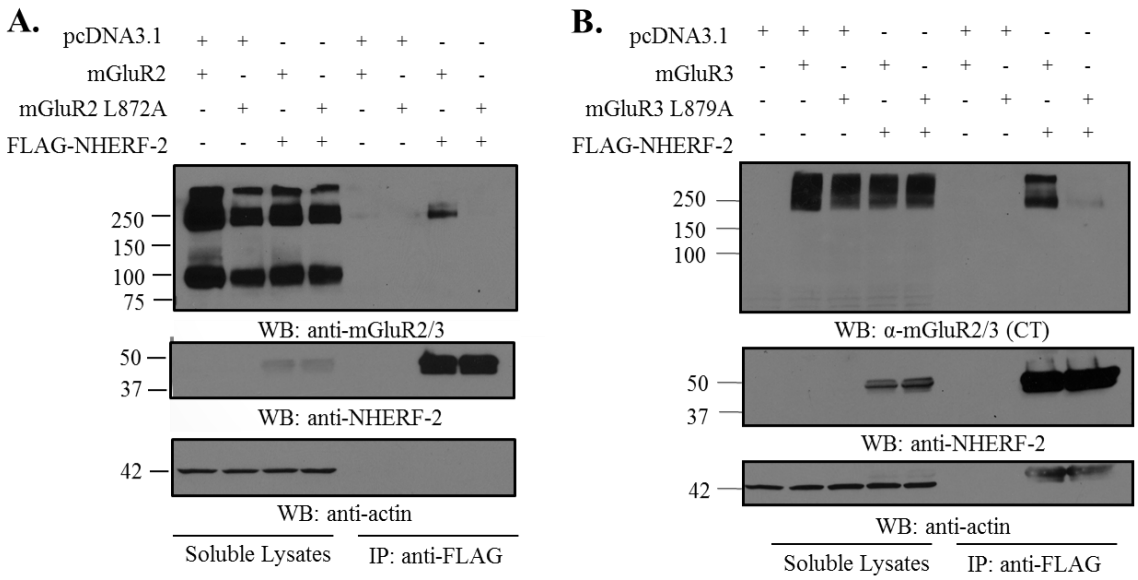


Figure 3.3. Group II mGluR association with NHERF proteins is dependent on the last amino acid. HEK293T cells were transfected with pcDNA3.1, mGluR2, mGluR2 L872A, mGluR3, mGluR3 L879A, or FLAG-NHERF-2. **A)** Robust immunoprecipitatoin of NHERF-2 with FLAG agarose (middle panel, lanes 7 and 8) resulted in the co-immunoprecipitation of mGluR2 (top panel, lane 7), while mutation of the last amino acid of mGluR2 L872A (Leucine→Alanine) prevented NHERF-2 assocation (top panel, lane 8). These results represent two independent experiments. **B)** Robust immunoprecipitatoin of NHERF-2 with FLAG agarose (middle panel, lanes 7 and 8) resulted in the co-immunoprecipitation of mGluR3 (top panel, lane 7), while mutation of the last amino acid of mGluR3 L879A (Leucine→Alanine) nearly completely abolished NHERF-2 assocation (top panel, lane 8). These results represent three independent experiments. Similar results were obtained for Group II mGuR interaction with NHERF-1 (data not shown).

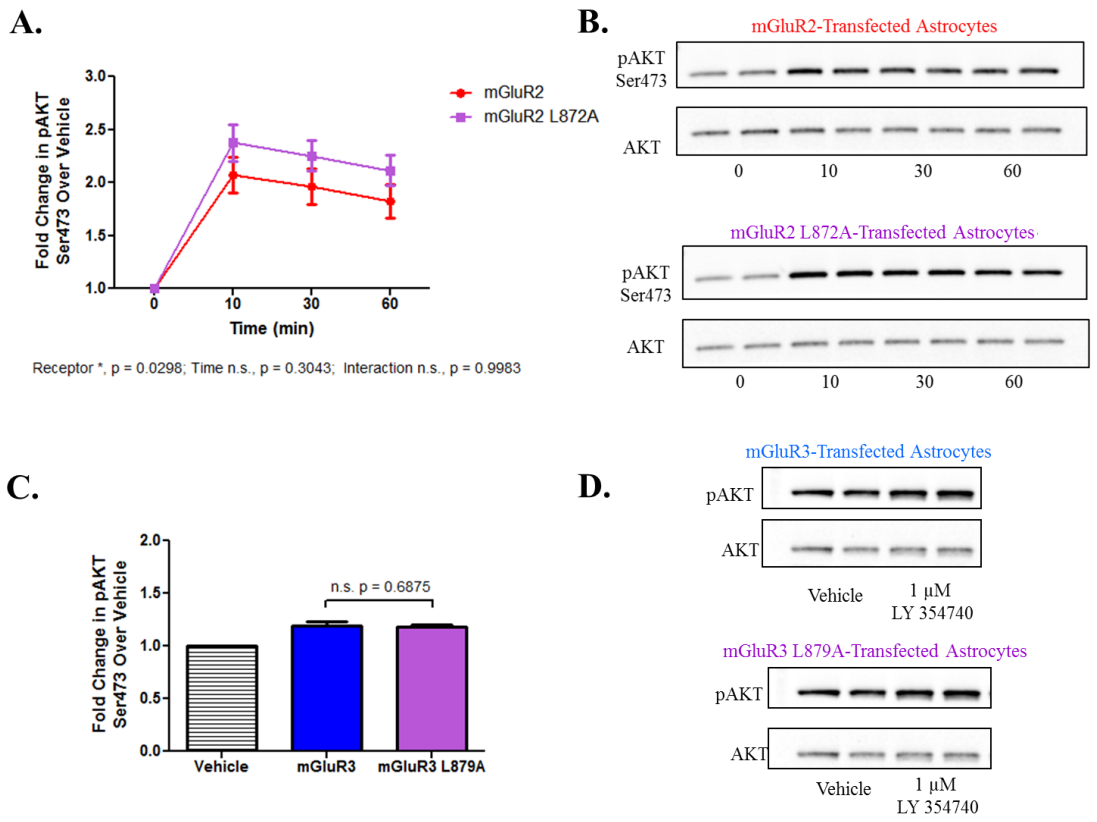


Figure 3.4. Examination of the effects of mutation of the Group II mGluR-PDZ interacting motif on AKT signaling. Mouse cortical astrocyte cultures expressing mGluR2 or mGluR2 L872A **(A and B)** and mGluR3 or mGluR3 L879A **(C and D)** were stimulated with either vehicle (media) or 1 µM LY 354740 for designated time-points. Astrocyte lysates were simultaneously probed for pAKT (Ser473) and total AKT. Graphs depict average fold change ± S.E.M. of normalized pAKT/AKT integrated densities over vehicle treatment. **A)** Analysis of mGluR2- and L872A-mediated AKT signaling via two-way ANOVA revealed an overall significant effect of receptor on signaling (n = 8, p = 0.0298, *). Enhanced mGluR2 L872A signaling was observed 6 out 8 times. **B)** A representative immunoblot showing the agonist-dependent activation of pAKT. **C)** A student's t-test revealed there was no significant difference between mGluR3- and L879A-mediated AKT signaling (n = 8). **D)** A representative immunoblot showing the comparably small agonist-dependent activation of pAKT.

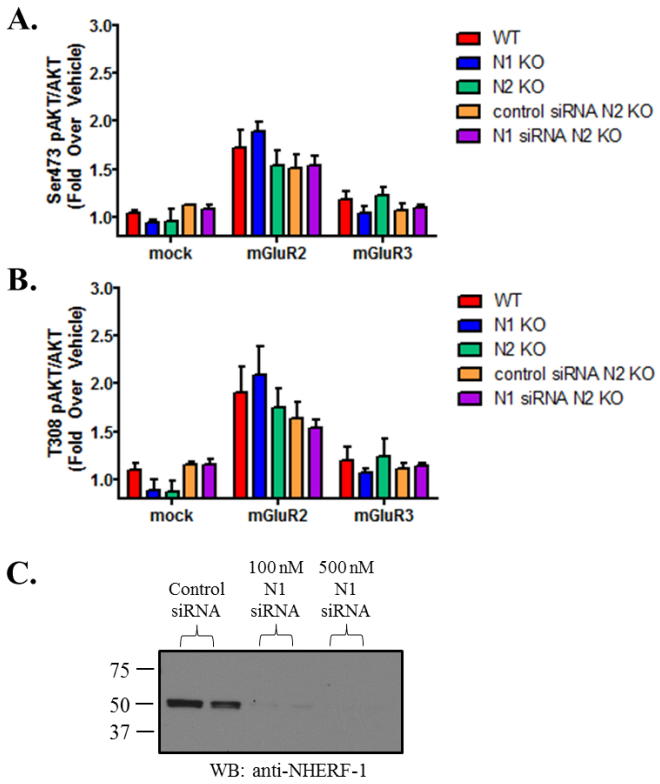


Figure 3.5. Examination of mGluR2 and mGluR3 signaling in cultured astrocytes devoid of NHERF proteins. Cortical astrocytes were prepared from WT, NHERF-1 KO, NHERF-2 KO, or NHERF-2 KO cultures treated with 250 nM control siRNA or NHERF-1 siRNA. Cultures were then transfected with mock (pcDNA3.1), mGluR2, or mGluR3 cDNAs and after 24 hours of expression and 3 hour serum starvation, were stimulated with either vehicle or 1 µM LY 354740 for ten minutes. Graphs depict normalized pAKT/AKT integrated densities ± S.E.M. Analysis of the activation of Ser473 AKT **(A)** or Thr308 **(B)** by Two-Way ANOVA revealed no significant effect of genotype, **(A)** $p = 0.86$, n.s. or **(B)** $p = 0.89$, n.s. However, a significant effect of receptor was observed, in accordance with the differential ability of mock-, mGluR2-, or mGluR3-transfected astrocytes to activate AKT, **(A)** $p < 0.0001$, *** or **(B)**, $p < 0.0001$, ***. These data are representative of 3-5 independent experiments per condition. **C)** A Western blot illustrating the dose-dependent knock-down of NHERF-1 with siRNA treatment. Cells were lysed 72 hours after treatment with NHERF-1 siRNA and probed for NHERF-1 levels.

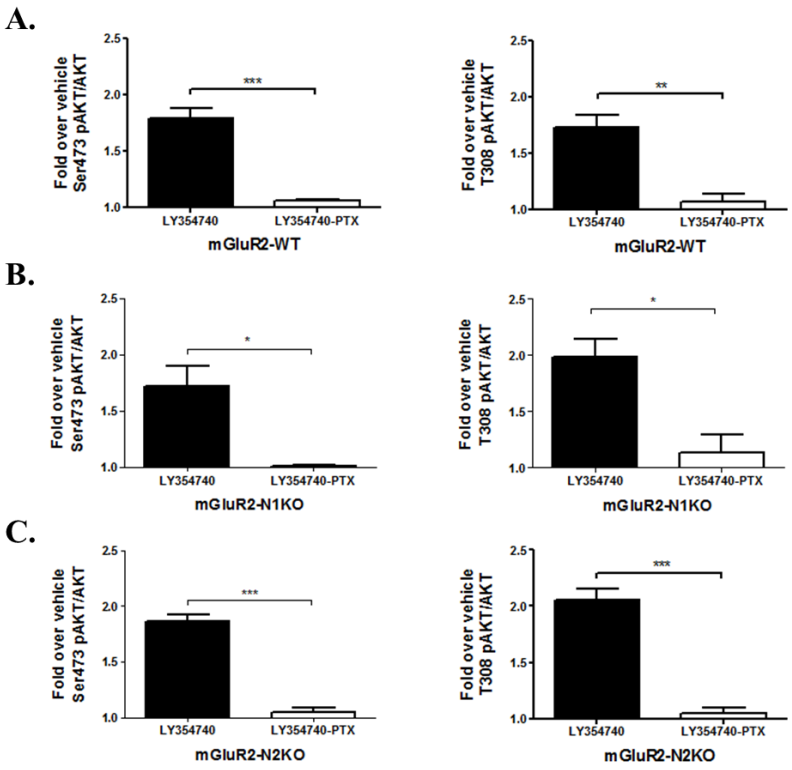


Figure 3.6. mGluR2-mediated AKT signaling is entirely mediated via G*α*i in WT, NHERF-1 KO and NHERF-2 KO astrocyte cultures heterologously expressing mGluR2. Heterologous expression of mGluR2 in astrocyte cultures prepared from WT, NHERF-1 KO, or NHERF-2 KO mice resulted in comparable stimulation of downstream AKT activaiton of Ser473 **(A)** and T308 **(B)**. Overnight treatment of cultures with pertussis toxin (PTX) abrogated agonist-dependent increases in AKT activation, as assessed via student's t-test. These data are representative of 3-4 independent experiments.

3.4. Discussion

Screening of Group II mGluR C-termini against a PDZ proteomic array revealed the NHERFs as candidate Group II mGluR-interacting partners, which expands upon the glutamate transporter, GLAST, (Ritter et al., 2011) and other metabotropic glutamate receptor, mGluR5, (Paquet et al., 2006a) as components of the astrocytic glutamate signaling system that NHERFs are known to bind to and regulate. Both mGluR2 and mGluR3 can associate with either NHERF-1 or NHERF-2 in cells, with a higher fraction of cellular mGluR3 (relative to mGluR2) being co-immunoprecipitated with the NHERF proteins. Mutation of the last amino acid of mGluR2 or mGluR3 disrupts NHERF-1 and NHERF-2 association, thereby providing insight into the structural determinants of the interactions.

In order to characterize how interaction with NHERF proteins could regulate Group II mGluR signaling, we examined the most robust signaling pathway that we could observe downstream of Group II mGluR activation. Using a strategy whereby Group II mGluR signaling is studied in cells expressing high levels of the glutamate transporter, GLAST, (Schoepp et al., 1997) we cultured astrocytes, which are known to have high levels of both NHERF proteins and GLAST (see Chapter 2; (Ritter et al., 2011). Additionally, primary astrocytes have been reported to express significant levels of mGluR3 under the conditions in which they are grown in certain other labs (Aronica et al., 2003; Bruno et al., 1998; Bruno et al., 1997; Ciccarelli et al., 2007; D'Onofrio et al., 2003; D'Onofrio et al., 2001; Durand et al., 2010). However, in our studies, we did not detect any endogenous mGluR3 protein via Western blot analysis, which is consistent with reports from other investigators in which mGluR3 mRNAs were detected in primary and/or secondary astrocyte cultures but evidence of mGluR3 protein was either equivocal or lacking (Aronica et al., 2003; Bruno et al., 1998; Bruno et al., 1997; Ciccarelli et al., 2007; D'Onofrio et al., 2003; D'Onofrio et al., 2001). Furthermore, we did not observe any downstream functional consequences of activation of Group II mGluRs in our cultured astrocytes, as measured by

accumulation of intracellular cAMP (data not shown), activation of pERK or activation of AKT (see Appendix, Figure A.3). In contrast, transfection of mGluR2 or mGluR3 into cultured cortical astrocytes resulted in a measureable increase in AKT activation that was mediated by Group II mGluR agonists, although no consistent effect was observed for activation of ERK (see Appendix, Figure A.3). Expression of mGluR2 has been reported in certain populations of astrocytes (Phillips et al., 2000), but many other studies have reported that mGluR2 expression is quite low or undetectable in the majority of brain astrocytes (Neki et al., 1996; Tanabe et al., 1992). Nonetheless, in order to compare how NHERF proteins could regulate both mGluR2 and mGluR3, we compared the two receptors in the same cellular context.

Initially, we found that disruption of direct C-terminal PDZ proteins with mGluR2 enhances mGluR2-mediated AKT signaling, while having no significant effect on mGluR3-mediated AKT signaling. The PDZ-disrupting mutation can block associations with the NHERF proteins and also probably block interactions with additional C-terminal binding partners of mGluR2/3 such as tamalin (Kitano et al., 2002), PICK1 (Hirbec et al., 2002), and GRIP (Hirbec et al., 2002), in addition to the additional candidate Group II mGluR interacting proteins identified in the PDZ array. To complement this approach and determine how the NHERF proteins might influence receptor-mediated AKT signaling, studies of Group II mGluR signaling in astrocyte cultures prepared from WT, NHERF-1 KO, NHERF-2 KO, and NHERF-2 KO cultures treated with NHERF-1 siRNA were performed. Given our initial data, we had hypothesized that loss of one or both NHERF proteins would lead to an enhancement of Group II mGluR-mediated AKT signaling. However, no differences were observed for either Group II mGluR in their ability to signal to AKT.

It may be that constitutive and global loss of either NHERF protein leads to developmental changes in the cell that lead to perturbations in AKT signaling. A number of studies have demonstrated that NHERF proteins can attenuate tyrosine kinase-receptor mediated

AKT responses (Pan et al., 2008; Takahashi et al., 2006), arguing that this regulation stems from the ability of the NHERF proteins to associate with two negative brakes of the AKT signaling pathway the tumor suppressor protein, PTEN, and the phosphatases PHLPP1 and PHLPP2, which are responsible for dephosphorylating AKT at reside Ser473. Thus, if loss of the NHERF proteins leads to general distortions in cellular AKT signaling, it may be difficult to detect the specific effects of blocking NHERF interactions with Group II mGluRs. Finally, as the magnitude of the mGluR2, and even more so for the mGluR3, response itself is quite small, it may be that it is less amenable to asking questions as to how the NHERF proteins can regulate receptor-mediated signaling, as the signal to noise is small, making variability a larger concern.

In summary, these data from the primary astrocyte studies reveal an important role for the PDZ-interacting motif on the mGluR2-CT in regulating receptor signaling to AKT, but it is unclear whether interactions with the NHERF proteins or other C-terminal binding partners are the relevant associations being disrupted. One major complication in these signaling studies was the persistent need to transfect mGluR2/3 into our cultured astrocytes; it may have been easier to discern the effects of knocking out the NHERF proteins signaling mediated by endogenous Group II mGluRs. However, although the expression of mGluR3 in astrocytes has been well-documented using *in vivo* approaches, such as *in situ* hybridization studies (Tanabe et al., 1993; Testa et al., 1994) or immunohistochemical approaches (Muly et al., 2007; Ohishi et al., 1994; Petralia et al., 1996; Sun et al., 2013; Tamaru et al., 2001), the expression of Group II mGluRs in cultured astrocytes has been more controversial. Studies of cultured human astrocytes, as well as in astrocytoma and glioblastoma cell lines have consistently revealed that mRNAs for mGluR3 and mGluR5 can be detected, while mRNAs for mGluR1 and mGluR2 are generally not detected (Aronica et al., 2003). Moreover, comparison of the expression of mGluR3 and mGluR5 mRNA levels in astrocyte cultures grown in the absence of serum versus an astrocyte defined medium containing additional supplements and growth factors, reveals that mRNA levels are increased in

the astrocyte defined medium supplemented with exogenous basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Aronica et al., 2003). However the ability to detect mGluR3 via Western blot analysis reportedly has been more challenging (Aronica et al., 2003; Bruno et al., 1997; Ciccarelli et al., 1997). Although the astrocyte-defined medium supplemented with exogenous growth factors increased mGluR3 and mGluR5 mRNAs, this increase was only mirrored at the protein level for mGluR5 in cultured astrocytes or U-373 astrocytoma cells as assessed via immunocytochemistry (Aronica et al., 2003). Moreover, Western blot analysis of human astrocyte cell lysates has been shown to be sufficient to detect mGluR5 (30 μg protein sample), while mGluR3 (even 150 μg cell lysate sample) was equivocally detected (Aronica et al., 2003). More recently, certain groups have suggested that they can detect mGluR3 via Western analysis approaches in their cultures (Durand et al., 2010); and furthermore that treatment with lipopolysaccharide (LPS) can induce protein expression of mGluR3 (Durand et al., 2010). In future work it will be of interest to determine why the expression of Group II mGluRs in cultured astrocytes can be so variable depending on culturing conditions.

3.5. Materials and Methods

Overlay of PDZ Array: Fusion proteins were purified and overlays of the PDZ domain array were performed as previously described (Fam et al., 2005; He et al., 2006). Briefly, 1 μg of His- and S-tagged PDZ domain fusion proteins were spotted onto nitrocellulose, dried overnight, and then overlaid with GST-alone (control), GST-mGluR2-CT, or GST-mGluR3-CT. Membranes were washed and incubated with an HRP-coupled anti-GST monoclonal antibody (Amersham Pharmacia Biotech) and binding of GLAST-CT fusion protein was visualized using enhanced chemiluminescence.

Cell Culture: HEK293T and astrocyte cultures were maintained in Glutamax™ DMEM (Invitrogen) containing 5% dialzyed FBS and 1% pen/strep in a humidified incubator at 37ºC

with 95% air and 5% CO_2 to regulate pH. Cortical secondary astrocyte cultures were prepared from neonatal mice (P0-P2) in the method of McCarthy and Vellis (McCarthy and de Vellis, 1980) with minor modifications. The day after plating astrocytes, cultures were vigorously shaken to dislodge contaminating cell types and media was replaced. Every three to four days media was changed and flasks would be manually shaken each time astrocytes were passaged. After primary cultures reached confluence, cultures were passaged using trypsin and grown to confluence again, where they were then split into wells for signaling studies. These "tertiary" astrocytes were typically used after 25 -30 days in culture for experiments.

Transfection: Mock pcDNA3.1+ (Invitrogen), rat mGluR2 or mGluR3 (originally in pBK-CMV and provided by Jeff Conn, Vanderbilt University; subcloned into pcDNA3.1), human FLAG-NHERF-1 (pBK-CMV) and rabbit FLAG-NHERF-2 (pBK-CMV) cDNAs were verified by sequencing. For HEK293T studies, Lipofectamine 2000 was used for transient transfections in accordance with manufacturer's instructions. Endotoxin-free cDNAs were used with TransIT®-LT1 (Mirus) transfection reagent for all astrocyte experiments in accordance with the manufacturer's instructions. In brief, a total of 2.5 µg of cDNAs were transfected per well of a 6-well plate. DNA complexes were mixed with opti-MEM supplemented with Glutamax™ and the TransIT-LT1 reagent at a 3:1 ratio for 20 minutes prior to drop-wise addition to confluent astrocyte cultures. Experiments were performed 24-48 hours after transient transfection.

Site-Directed Mutagenesis: Mutagenesis of the last amino acid of the mGluR2 or mGluR3 was accomplished in accordance with the manufacturer's instructions, using Quikchange Site-directed mutagenesis (Stratagene). Primers used for mGluR2 L872A were forward 5' GAC TCA ACA ACG TCG TCG GCT TGA AGA TCC CAC ACT CC 3' and reverse 5' GGA GTG TGG GAT CTT CAA GCC GAC GAC GTT GTT GAG TC 3'. Primers used for mGluR3 L879A were forward 5' GAC TCC ACC ACC TCA TCT GCG TGA CTC GA 3' and reverse 5' CCT CGA

GTC ACG CAG ATG AGG TGG TGG A 3'. Primes were purchased from integrated DNA technologies (IDT) and were PAGE purified. All constructs were verified using sequencing.

Immunoprecipitation: Following transient transfection, cells were washed twice with ice-cold PBS supplemented with calcium to remove albumin. Cells were lysed with 1 ml of ice-cold harvest buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% triton-X-100, and 1 protease inhibitor cocktail tablet (cOmplete, EDTA-free, Roche)). Membrane proteins were solubilized at 4°C with end-over-end rotation for one hour and subsequently centrifuged at 13,000 RPM to pellet insoluble fraction. A sample of the soluble lysate was saved and diluted with 6X Laemmli buffer to a 1X concentration, while remaining soluble lysate incubated with FLAG-agarose (Sigma) for one to two hours to immunoprecipitate FLAG-tagged NHERF proteins. IP samples were sequentially washed three times with ice-cold harvest buffer and gentle vortexing and then eluted with 2X Laemmli buffer and left to denature for 18 to 24 hours prior to use. Samples from soluble lysates and immunoprecipitates were subjected to SDS-PAGE and Western analysis.

Astrocyte Stimulation for Signaling Studies: Six-well plates of tertiary astrocyte cultures were transiently transfected with endotoxin-free cDNAs encoding pcDNA3.1+, mGluR2, or mGluR3 as described above. After 24 hours, cells were washed three times and serum starved in 1 ml of incomplete Glutamax™ DMEM for 3-5 hours prior to stimulation with media (vehicle) or 1 μM LY354740 (Tocris) dissolved in 100 mM NaOH to neutralize the acid and diluted in incomplete media to a final 2X concentration. Application of 1ml of this 2X drug was applied to cells that had been starved in 1 ml of media, so that the drug was a final 1X concentration. This method of drug application was found to not induce erroneous increases in phospho-ERK activation, which were observed just by the aspiration and replacement of media (vehicle) within a well. After treatment, media was rapidly aspirated and cells were lysed in 2X Laemmli buffer. Samples were then sonicated and stored at -20°C if not needed.

SDS PAGE and Western Analysis: Prior to use, any frozen samples were thawed and vortexed to dissolve precipitated SDS. Proteins were then subjected to SDS-PAGE using 4-20% tris-glycine gels. Proteins were then transferred onto nitrocellulose. For AKT signaling studies, membranes were blocked using Odyssey blocking buffer (LiCor) and two florescent secondaries were used for simultaneous probing of phospho- and total AKT proteins. Primary antibodies used for AKT and ERK signaling studies were: rabbit monoclonal anti-phospho AKT (Ser473) XP clone D9E, (Cell Signaling Technology, catalog # 4060), rabbit monoclonal anti-phospho AKT (Thr308), clone C31E5E, (Cell Signaling Technology, catalog # 2965), mouse monoclonal total anti-AKT (pan), clone 40D4, (Cell Signaling Technology, catalog # 2920), rabbit monoclonal total anti-ERK 1/2, (Cell Signaling Technology, catalog # 9102), and mouse monoclonal anti-phospho ERK, clone E-4, (Santa Cruz Biotechnology, catalog # sc-7383). Secondary antibodies used for AKT and ERK signaling studies were: anti-mouse or anti-rabbit Alexa Fluor 680 (Sigma) and anti-mouse or anti-rabbit IR-dye 800CS (Li-Cor). Primaries used for immunoprecipitation and/or expression studies were: rabbit polyclonal anti-mGluR2/3 (Chemicon, catalog # 06-676), rabbit polyclonal anti-NHERF-1 (Ab 5199, generously provided by C. Chris Yun) or rabbit polyclonal anti-NHERF-2 (Ab 2570, generously provided by C. Chris Yun), and anti-actin antibodies rabbit monoclonal anti-actin (Sigma, catalog # A2066). Following 1 hour incubation at RT, membranes were washed for 3 x 5 minutes and then incubated with the corresponding HRP-conjugated secondary (GE Healthcare, ECL™ anti-mouse or rabbit IgG, HRP-linked, whole antibody) for 30 minutes. The membrane was then washed 3 x 10 minutes and rinsed once with deionized water. Bands were visualized using an enhanced chemiluminescence kit (Pierce) and exposed to films for various time-points. The membrane was then stripped with Restore Buffer (Pierce) and probed with rabbit anti-actin (Sigma) as an internal control for protein loading.

C57BL6J (WT), NHERF-1 KO, and NHERF-2 KO mice: All mice were treated in accordance with IACUC guidelines at Emory University. Both NHERF-1 and NHERF-2 KO mice are congenic on C57BL6J (JAX). NHERF-1 KO mice were originally created by Ed Weinman (University of Maryland) via a neomycin insertion into exon 1, while NHERF-2 KO mice were originally created by lexicon genomics via a lentiviral gene trapping cassette (clone OST2298) found to be inserted into the intronic region after exon 2 and provided to us via C. Chris Yun (Emory University). Primers were used to genotype mice and set-up breeders.

Genotyping: In accordance with IACUC guidelines, tail clips were taken from mice at P11 to P14 postnatal. Tails were then digested with a protease buffer (Proteinase K 2 mg/ml, SDS, EDTA, NaCl) overnight at 55°C and subsequently mixed with isopropranol to precipitate DNA. Approximately 500 ng/ml of DNA were used for each PCR cycle. Primers for NHERF-1 were originally developed by (Shenolikar et al., 2002). They are P1 (Common Forward): 5' CTC TGT TTA TTC CCA GAA GGA 3'; P2 (Neomycin Cassette, Mutant): 5' CAA GAA GGC GAT AGA AGG CGA TG 3'; and P3 (WT Reverse): 5' GAG CCA GGT TCT ACC AGA CGG ATA AAC TGG 3.' PCR results were run on a 0.8% agarose gel with ethidium bromide to reveal PCR products. Primer combinations of P1 and P2 yielded a 2400 bp fragment that corresponded with the NHERF-1 mutant allele, while primer combinations of P1 and P3 yielded a 1400 bp fragment that corresponded with the WT NHERF-1 allele. The primers for NHERF-2 were originally developed by the Yun lab (Singh et al., 2009). They are P1 (Common Forward): 5' TTC TAT AAG CCT CCA TTT CCT CT 3'; P2 (WT Reverse): 5' CCC ACC CCC ATC GCT GCT C 3'; and P3 (Mutant Reverse): 5' GCG CCA GTC CTC CGA TTG A 3'. PCR results were run on a 2.0% agarose gel with ethidium bromide to reveal PCR products. Primer combinations of P1 and P2 yielded a 303 bp fragment corresponding with the NHERF-2 mutant allele, while primer combinations of P1 and P3 yielded a 229 bp fragment corresponding to the WT NHERF-2 allele.

Negative control reactions in which the DNA was not included were also run to control for contamination.

siRNA Knockdown: Control siRNAs (Silencer Negative Control #1) and NHERF-1 silencer select siRNAs were purchased from Ambion, Life Sciences technologies (catalog # 4390771, targets mouse exon 1). siRNAs stocks were reconstituted at 100 μM and dissolved in PCR-grade water, and stored at -80°C, until needed. In accordance with the manufacturer's instructions, cultured astrocytes were nucleofected with either control or NHERF-1 siRNA, using the AMAXA Basic Mammalian Glial Cells kit (Lonza). Following 72 hours of expression, astrocytes were lysed and examined for NHERF-1 and NHERF-2 levels. Signaling studies were also performed at the 72 hour time point.

3.6. Acknowledgements

I would like to thank Drs. Ed Weinman and C. Chris Yun for donation of the NHERF-1 and NHERF-2 KO mice towards the start of our own colonies, respectively. I would like to thank Songbai Lin for assistance with primer design for the NHERF-2 KO mice and William Watkins for assistance with managing the NHERF-1 and NHERF-2 mouse colonies. I would like to thank Christopher Makinson for guidance on DNA extraction from mouse tails and genotyping. I would like to thank the Kukar lab for letting me use their Li-COR for the Western blot analysis.

CHAPTER 4

CNS Characterization of NHERF-1 and NHERF-2 KO mice

4.1. Summary

The previous chapters have documented NHERF protein associations *in vitro* with the astrocytic glutamate transporter GLAST and the Group II metabotropic glutamate receptors (mGluRs) mGluR2 and mGluR3. However, nothing is known about the potential *in vivo* significance of these interactions. Given the presence of the ezrin-radixin-moesin (ERM) binding domain on the NHERF proteins, we hypothesized that the NHERF proteins might be critical regulators of GLAST and Group II mGluR trafficking *in vivo*, potentially influencing GLAST and mGluR overall expression levels and targeting GLAST and the mGluRs to ezrin-enriched perisynaptic astrocyte processes (PAPs). However, loss of either NHERF-1 or NHERF-2 did not change total expression levels of GLAST or Group II mGluRs in the cortex, striatum, hippocampus, or cerebellum, as compared to brain homogenates from wild-type (WT) age-matched and sex-matched mice. Electron microscopic analyses of the cellular and subcellular localization of Group II mGluRs in the cortex across WT, N1 KO, and N2 KO mice revealed that Group II mGluRs are primarily distributed to glia and unmyelinated axons in WT and N1 KO mice. However, a shift in the relative distribution of Group II mGluR labeling in N2 KO mice was observed, which corresponded to increased Group II mGluR immunoreactivity in unmyelinated axons. Furthermore, Group II mGluR labeling in PAPs, was quantified and found to not vary across groups. These studies reveal a surprising role for NHERF-2 in influencing the targeting Group II mGluRs to pre-terminal, unmyelinated axons, but no evident role for the NHERF proteins in controlling the targeting of Group II mGluRs to PAPs. Other interesting observations made during the course of these studies included the characterization of *i)* a previously-uncharacterized putative NHERF-2 splice variant, containing one PDZ domain and the ERM binding domain, which was upregulated in the NHERF-2 KO (N2 KO) mice relative to WT and NHERF-1 KO (N1 KO) mice, and *ii)* robust hydrocephaly in a fraction of the N1 KO mice, as well as ventriculomegaly in N1 KO and N2 KO mice, relative to the littermate controls,

suggesting a role for NHERF-1, and possibly NHERF-2, in the control of fluid homeostasis *in vivo* and the etiology of hydrocephaly.

4.2. Introduction

The Na+/H+ Exchanger Regulatory Factors 1 and 2 (NHERF-1 and NHERF-2) have been shown to be localized within the rodent brain in neurons (Lee et al., 2007a; Paquet et al., 2006b), astrocytes (Lee et al., 2007a; Paquet et al., 2006b), endothelial cells (Lee et al., 2012; Lee et al., 2007a; Paquet et al., 2006b), pericytes (Paquet et al., 2006b) and choroid plexus epithelial cells (Lee et al., 2012), albeit to varying degrees across cell types. However, little is known about the functional significance of the NHERF proteins in the mammalian brain *in vivo*. Additionally, much work has been done to examine the effects of the NHERF proteins on glutamate transporter and Group II metabotropic glutamate receptor (mGluR) function *in vitro*. However, these findings, as presented in Chapters 2 and 3, need to be put into a broader context in order to examine if there is any significance of the interactions *in vivo*. To this end, NHERF-deficient mice were acquired from the laboratories of Ed Weinman (NHERF-1 KO mice) and C. Chris Yun (NHERF-2 KO mice).

Many of the reported phenotypes of the NHERF-1 KO mice have focused on alterations in urinary secretion of phosphate (Shenolikar et al., 2002), calcium and uric acid (Cunningham et al., 2007), as well as alterations in sodium proton exchanger (NHE3) function in the kidney (Shenolikar et al., 2002). Additionally, there is a brief mention in the literature about observations of hydrocephaly in NHERF-1 KO females on the C57BL6/J background (Shenolikar et al., 2002), although this was not quantified and moreover was not observed in NHERF-1 KO mice on the FVB/n background (Broere et al., 2007). The reported phenotypes for the NHERF-2 KO mice include alterations in the morphology of the intestines (Murtazina et al., 2011), as well as enhanced forskolin-stimulated intestinal HCO_3^- secretion (Singh et al. 2007). However, the

increased urinary phosphate and uric acid secretion observed in the NHERF-1 KO mice was absent in NHERF-2 KO mice (Cunningham et al., 2008). To date, no thorough CNS characterization has been performed on the NHERF-1 or the NHERF-2 KO mice, despite the fact that the NHERFs have been found to associate with a number of proteins found in the central nervous system including the astrocytic glutamate transporter GLAST (Lee et al., 2007a; Ritter et al., 2011), Group II mGluRs, mGluR5 (Paquet et al., 2006a), the platelet-derived growth factor beta receptor PDGFβ (Maudsley et al., 2000), the purinergic receptor $P2Y_1$ (Fam et al., 2005; Hall et al., 1998a), the kappa opioid receptor kOPR (Li et al., 2002), and the chloride channel CLC-5 (Hryciw et al., 2006). The goal of the studies described in this chapter was to shed light on the physiological importance of NHERF proteins in the mammalian central nervous system, with a focus on assessing potential NHERF regulation of glutamate transporters and metabotropic glutamate receptors. To this end, assessments of brain pathology were performed and the ultrastructural localization patterns of Group II mGluRs were studied in cortical brain tissue derived from WT, NHERF-1 KO, and NHERF-2 KO mice.

4.3. Results

Three primer PCR was used to genotype NHERF-1 and NHERF-2 mice (Figure 4.1, panel A), similar to previously reported approaches (Broere et al., 2007; Shenolikar et al., 2002). In order to validate that the NHERF KO mice were actually deficient in NHERF proteins, we examined NHERF-1 and NHERF-2 expression levels in various brain regions of the mice using polyclonal antibodies that have been extensively characterized, (Lamprecht et al., 1998; Yun et al., 1998). Full-length NHERF-1 and NHERF-2 were not detected in brain lysates from NHERF-1 KO or NHERF-2 KO mice (Figure 4.2B). Additionally, total levels of the full-length NHERFs did not change in the respective NHERF KO mouse brain samples, suggesting there was not a compensatory upregulation of one NHERF protein, when the other was knocked out.

Interestingly, the NHERF-2 antibody detected a novel band in the NHERF-2 KO brain tissue samples. This band migrated on SDS-PAGE gels at approximately 27 kDa and exhibited a distinctive pattern of expression across the three genotypes studied: it was significantly increased in all three NHERF-2 KO mouse brain samples and slightly decreased in all three NHERF-1 KO mouse brain samples, relative to WT samples.

We hypothesized that this band may be a splice variant of NHERF-2. Indeed, Genbank searches revealed reports of a NHERF-2 transcript variant that is present in both mouse and humans and encodes a truncated version of NHERF-2 that lacks the second PDZ domain and is predicted to be 24.5 kDa in mass. Analyses of the NHERF-2 full length mRNA and the NHERF-2 splice variant revealed them to differ in the intronic region in which the NHERF-2 retroviral gene-trapping cassette was initially inserted (Lexicon Genetics, personal communication). Thus, the NHERF-2 splice variant would not have been targeted by the gene trapping method, which supports the idea that the observed 27 kDa band that is upregulated in the NHERF-2 KO brain tissue is indeed the protein product of the shorter NHERF-2 transcript variant.

Since the expression of this splice variant at the protein level has never been reported, we examined whether it might be expressed in other tissue types. Six different tissue lysates from WT and NHERF-2 KO mice were examined, including small intestine, liver, kidney, brain, heart, and smooth muscle (Figure 4.1C). The full length NHERF-2 was absent from all KO tissues examined; however, the 27 kDa band corresponding to the splice variant was upregulated in 5 out of 6 of the tissues examined. The expression of the band corresponding to the splice variant was highest in the kidney and low in the brain, relative to the expression level of full-length NHERF-2 in the WT tissue.

Following this initial characterization of the brain tissue from the NHERF KO mice, we sought to examine if expression levels of NHERF-interacting partners might be altered in the KO brain tissue. Chapters 2 and 3 explored the functional roles that NHERF proteins can have on

glutamate transporter and glutamate receptor function. Given that NHERF-2 was shown in the experiments described in Chapter 2 to enhance GLAST expression and thereby promote its function in cultured astrocytes, we hypothesized that the absence of NHERF-1 and/or NHERF-2, might lead to a reduction in GLAST levels in the NHERF-2 KO mice, relative to WT mice. To this end, we examined GLAST expression levels in three different brain regions, cortex, hippocampus, and striatum, in WT, NHERF-1 KO, and NHERF-2 KO mice. As shown in Figure 4.2, GLAST levels were not significantly changed across genotypes in cortex, hippocampus, striatum, or cerebellum as assessed via Western blot analysis. Additionally, we examined if Group II mGluR levels were altered across NHERF genotypes and found that Group II mGluR total expression was relatively stable across all three genotypes for all four brain regions examined (cortex, hippocampus, striatum, and cerebellum) (see Figure 4.3). These two data sets together support that loss of one of the full-length NHERF protein has no effect on the global expression of GLAST or Group II mGluRs. As we were unable to successfully generate double NHERF-1/-2 KO mice on the C57BL6/J background (see section below on hydrocephaly), we could not examine if developmental and constitutive loss of both NHERF proteins had effects on glutamate transporter and metabotropic receptor expression.

Even if NHERF proteins do not change the total expression levels of GLAST or Group II mGluRs in the examined brain regions, it is still possible that loss of either or both of the NHERF proteins may alter the cellular localization of these proteins. To address this question, we used immuno-electron microscopy (EM) to characterize the localization of Group II mGluRs in the brain. At the time that these studies were performed, commercially-available anti-GLAST antibodies suitable for EM did not exist, as the original reports had relied on GLAST antibodies that were custom-made. In contrast, excellent antibodies were available to detect the Group II mGluRs and these antibodies had been recently used to examine Group II mGluR localization in the rodent brain at EM level (Lavialle et al., 2011; Muly et al., 2007). Therefore, we set out to

examine whether Group II mGluR distribution might be altered in NHERF-1 or NHERF-2 KO mice, relative to WT-mice littermate controls.

Light microscopy analysis of brain sections revealed that Group II mGluR immunoreactivity was widespread and predominantly observed in neuropil (unmyelinated axons, dendrites, spines, or glia processes), without labeling of neuronal cell bodies. The section of tissue selected for the reaction was anterior to the hippocampus and contained cortex, striatum, and some nucleus accumbens, where predominant cortical labeling had been observed. A similar section of tissue was chosen for all animals in order to compare the same cortical section across groups. To this end, a similar degree of labeling was observed across all groups, regardless of genotype (Figure 4.4). Moreover, light microscopic reactions of Grm2 or Grm3 KO mouse tissue revealed a loss of signal intensity, corresponding to loss of mGluR2 or mGluR3 immunoreactivity, respectively (data not shown). However, as the antibody detects both mGluR2 and mGluR3, some immunoreactivity remained that presumably represented the remaining mGluR receptor. In order to demonstrate the specificity of the Group II mGluR antibody, control reactions were done in parallel, whereby the Group II mGluR antibody was preadsorbed prior to incubation with the tissue. As shown in Figure 4.4, immunoperoxidase deposits were almost completely absent when the pre-adsorbed Group II mGluR antibody was used, relative to use of the Group II mGluR antibody. This was observed in one representative brain section that was chosen from each group. The pattern of labeling observed and control reaction is consistent with previous reports regarding the specificity of this antibody (Lavialle et al., 2011; Muly et al., 2007; Petralia et al., 1996). Additionally, although care was taken to sample similar tissue sections across all groups, sections from NHERF-1 and NHERF-2 KO mice had larger ventricles, relative to their WT littermates (Figure 4.4). Similarly, this was observed for all 6 NHERF KO mice used in this immuno-electron microscopy study. These results are consistent with the hydrocephaly phenotype observed in the NHERF-1 KO mice (see section below on hydrocephaly).

To characterize the cellular and subcellular distribution of Group II mGluR immunoreactivity in the cortex, single immunoperoxidase labeling for Group II mGluRs was performed across WT, NHERF-1 KO and NHERF-2 KO mice. Neuronal and glial elements were classified based on morphological features using criteria established by Peters et al. (Alan Peters, 1991) such as asymmetric shape, presence of glycogen granules, and relatively clear cytoplasm. Representative images of Group II mGluR labeling across WT (Figure 4.5 A-C), NHERF-1 KO (Figure 4.5 D-F), and NHERF-2 KO (Figure 4.5 G-I) are shown in Figure 4.5. As shown in Figure 4.6, quantification of these images across genotypes revealed that Group II mGluR labeling was primarily observed in glia and unmyelinated pre-terminal axons, with labeling less commonly seen in axon terminals or post-synaptic structures such as dendrites and spines. Interestingly, comparisons of the relative distribution of Group II mGluR labeling across genotypes indicated that WT and NHERF-1 KO mice had a comparable distribution of Group II mGluR immunoreactivity, with approximately 55% of the labeling corresponding to glia and 40% of the labeling corresponding to unmyelinated axons. In contrast, a shift in increased axonal versus glial Group II mGluR labeling was observed in NHERF-2 KO mice, relative to WT and NHERF-1 KO mice (Figure 4.6 A). Analyses of the densities (counts divided by area of tissue examined) across groups revealed that significantly more Group II mGluR labeled axons were detected in NHERF-2 KO mice, relative to WT or NHERF-1 KO mice (Figure 4.6 B). No appreciable differences in the total number of labeled glia were observed across the various genotypes, suggesting that the shift in the Group II mGluR distribution in the NHERF-2 KO mice corresponded to an increase in axonal Group II mGluR labeling and not a decrease in glial labeling. Additionally, quantification of the proportion of Group II mGluR-immunoreactive glia in the mouse cortex revealed that 47.5% ± 1.24% (mean ± S.E.M.) of all glia are labeled for Group II mGluRs, implying a significant function for Group II mGluRs in glia, which based on morphological criteria, presumably correspond to astrocytes.

Given the significant glial labeling observed in all groups and the fact that much of this labeling appeared to associate with asymmetric synapses and thereby correspond to perisynaptic astrocyte processes (PAPs), glial labeling was further sub-divided into perisynaptic or non-perisynaptic astrocyte processes. Astrocyte processes are known to be intimately associated with synapses; however variations in the extent of astrocyte coverage, as assessed in two-dimensional micrographs, were observed. Consequently, PAPs were defined into three categories, corresponding to what extent they associated with pre- and post-synaptic elements and their distance from the post-synaptic density specialization (Figure 4.7 A). The relative distribution of Group II mGluR-labeled perisynaptic processes versus non-perisynaptic processes was examined across groups. Few meaningful differences were observed (Figure 4.7 C) and an analysis of mean densities did not support any significant differences in any PAP or non-PAP distributions across genotypes (Figure 4.7 D). A similar lack of observed findings was seen when PAP categories were collapsed (Figure 4.7 E, Figure 4.7 F).

Finally, comparisons of labeled and non-labeled PAP-A were made across genotypes, corresponding to the most stringent classification of the PAPs, ultimately revealing that there were no significant differences in the mean densities of the proportion of Group II mGluR-labeled PAP-A versus non-labeled PAP-A across genotypes (Two-way ANOVA, genotype and labeling, n.s.). Although an analysis of the relative distribution of labeled versus non-labeled Group II mGluR PAP-A in WT mice revealed that approximately 38.76% ± 2.05 (mean ± S.E.M.) of PAP-A in WT cortex are Group II mGluR-immunopositive, while 50.54% ± 2.89 or 48.52% ± 1.56, corresponding to N1 and N2 KO identified PAP-A in cortex, respectively. However, since the mean densities were not statistically different, these differences in relative distribution are not meaningful. In summary, approximately 38% of identified PAP-A in WT mice are immunolabeled for Group II mGluRs, highlighting the functional role of these receptors in this anatomical compartment.

Despite the lack of any observed differences in the distribution of Group II mGluR labeling in the PAP compartment, it is nonetheless important to validate the criteria and PAP categories used in this analysis. Thus, as an additional control, the number of asymmetric synapses were tabulated across groups and found to be statistically indistinguishable (One-way ANOVA, Tukey's multiple comparisons, n.s.), with mean synapse densities per 100 μm^2 ± S.E.M. being 32.87 ± 1.90, 38.07 ± 5.30, and 31.87 ± 1.68, corresponding to counted synapses in WT, N1 KO, and N2 KO mice respectively. Sampling a comparable numbers of asymmetric synapses across groups validates the comparisons of the PAP counts, as the number of asymmetric synapses are integral to the criteria. Lastly, perisynaptic glial processes are known to vary within and across brain regions, as they are highly dynamic processes (Ventura and Harris, 1999). To this end, three-dimensional reconstructions of Group II mGluR-labeled PAPs were performed in order to examine glial coverage of asymmetric synapses across groups. As shown in Figure 4.8, Group II mGluR labeled glia are intimately associated with the synapse and nearly entirely cover the post-synaptic density. These data are preliminary, as only a single full reconstruction was performed, and thus represent a proof of concept for future experiments.

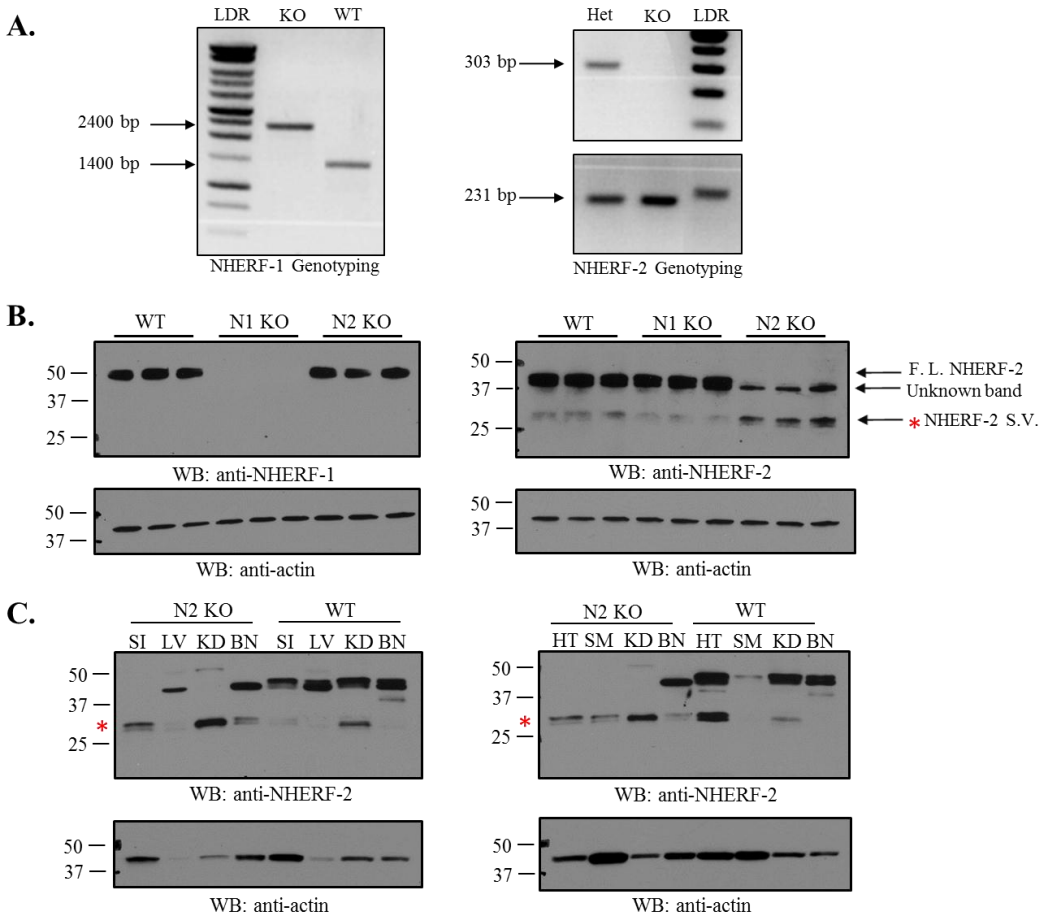


Figure 4.1. Characterization of NHERF genotype status and protein expression in NHERF-1 and NHERF-2 mutant mice: evidence for upregulation of a NHERF-2 splice variant. A) An example of PCR results for NHERF-1 and NHERF-2 genotyping of Wild-type (WT), Heterozygous, (Het) and knockout (KO) mice. The mutant NHERF-1 PCR product runs at 2400 bp, which includes the neomycin cassette, whereas WT NHERF-1 yields a 1400 bp fragment. The mutant NHERF-2 PCR product runs at 231 bp, reflecting the induced missplicing of the NHERF-2 transcript, whereas WT NHERF-2 yields a 303 bp fragment. **B)** Examination of protein levels of NHERF-1 and NHERF-2 in mouse brain tissue reveals that full length NHERF-1 is absent in NHERF-1 KO animals and its levels are unchanged in WT and NHERF-2 KO mice.

Similarly, full-length NHERF-2 is also absent in NHERF-2 KO mice and its levels are unchanged in WT and NHERF-1 KO mice. However, a presumed NHERF-2 splice variant (red asterisk) that is predicted to be 24.5 kDa and yet runs at approximately 27 kDa appears upregulated in NHERF-2 KO mice and its levels are slightly decreased in NHERF-1 KO mice, relative to WT mouse brain samples. The other band detected with the NHERF-2 antibody is unknown; it may reflect a non-specific band as this is a polyclonal antibody that is not affinity purified, or it may indicate another NHERF-2 splice variant. **C)** NHERF-2 tissue lysates from small intestine (SI), liver (LV), kidney (KD), brain (BN), heart (HT), and smooth muscle (SM) were examined and probed with the NHERF-2 antibody, A2725, via Western blot analysis. In all 6 tissues examined, full length NHERF-2 was absent; however, in 5 out of 6 of the tissues, excluding heart, the presumed NHERF-2 transcript variant (24.5 kDa) was upregulated (red asterisk). Interestingly, the unknown band that was detected in the brain in **(B)** was also apparently present in the liver as well. Although NHERF-2 appears to be detected as a doublet with this antibody, the doublet only disappeared in small intestine, kidney and heart.

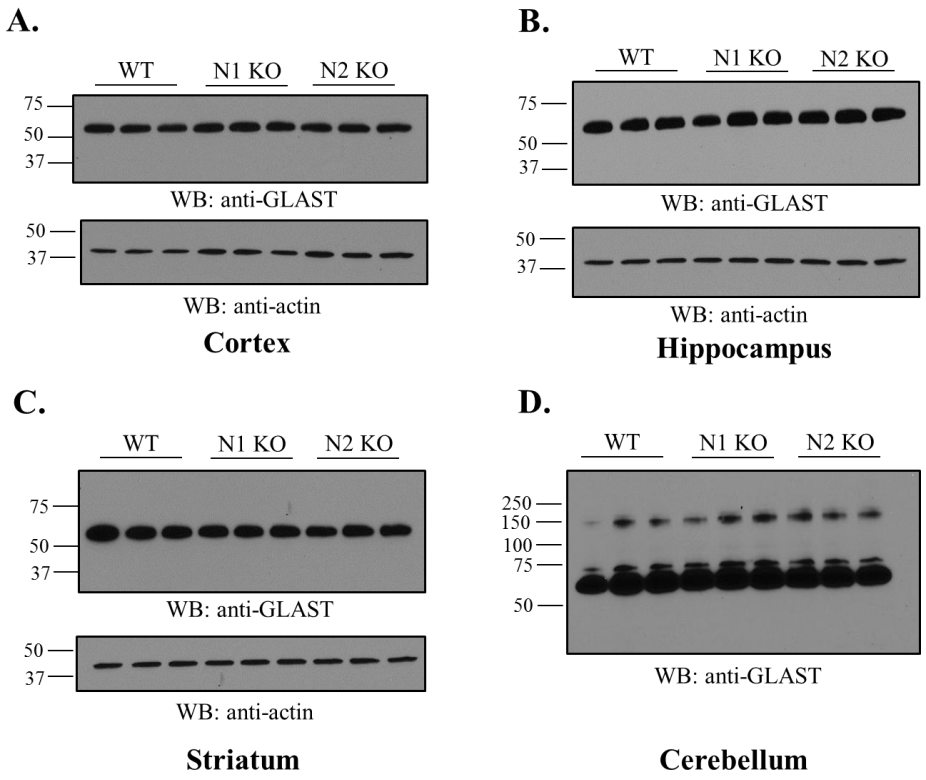


Figure 4.2. Total GLAST expression does not change in wild-type, NHERF-1 KO, or NHERF-2 KO mouse brain regions. A) Equal protein amounts (15 µg) of brain lysates were subjected to SDS PAGE and Western blot analysis for detection of GLAST and actin. No differences in GLAST expression levels were observed across genotypes, in either **(A)** Cortex, **(B)** Hippocampus, **(C)** Striatum, or **(D)**. Blots were stripped and re-probed with actin to confirm equal protein loading; however due to technical error, the actin blot is not available for the cereeballar samples **(D)**. However, examination of actin expression in Figure **4.3 D** demonstrates that cerebellar samples do have similar protein concentrations, as assessed by that actin blot.

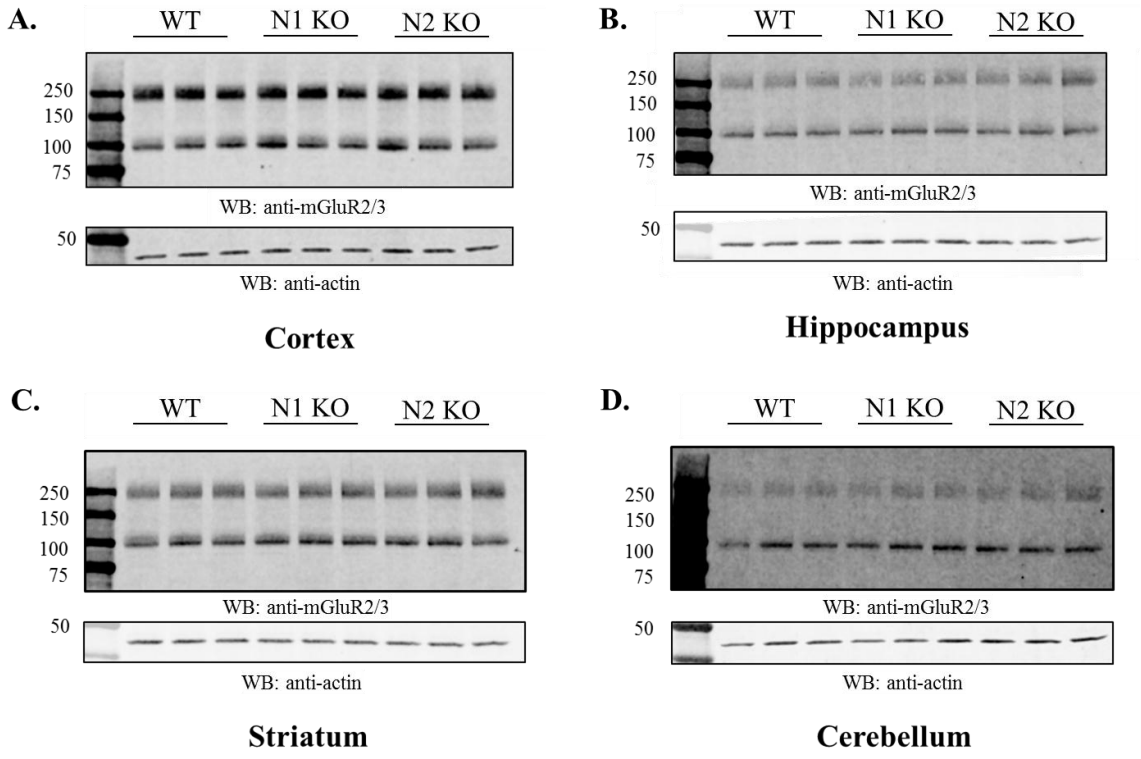


Figure 4.3. Total Group II mGluR expression does not change in wild-type, NHERF-1 KO, or NHERF-2 KO mouse brain regions. A) Equal protein amounts (10 µg) of brain lysates were subjected to SDS PAGE and Western blot analysis for detection of Group II mGluRs and actin. Blots were stripped and re-probed for actin to confirm equal protein loading. No differences in Group II mGluR expression levels were observed across genotypes, in either **(A)** Cortex, (**B)** Hippocampus, **(C)** Striatum, or **(D)** Cerebellum.

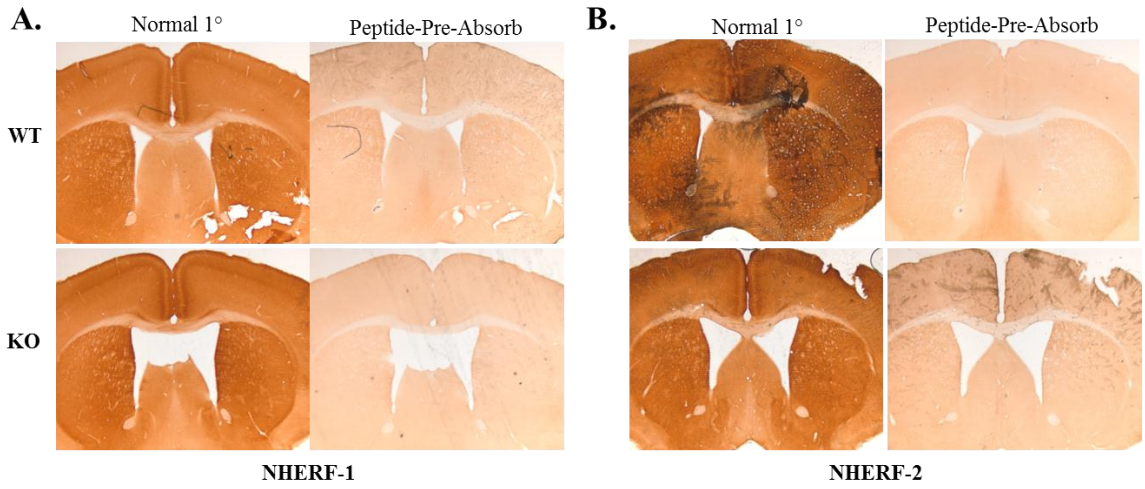


Figure 4.4. Control experiments for light microscopy reaction and evidence of ventriculomegaly in NHERF-1/-2 KO mice. Light microscopy reactions with Group II mGluR antibody (Ab1553) were done in parallel with an mGluR2-CT peptide that was pre-absorbed with the Group II mGluR antibody. Note absence of Group II mGluR signal in the peptide pre-absorbed conditions for all groups examined **(A)** WT and NHERF-1 KO littermates and **(B)** WT and NHERF-2 KO littermates. Additionally, all 6 NHERF-1 or NHERF-2 KO mice had enlarged ventricles relative to their littermate controls. Sections used in the staining were all selected from a similar brain region.

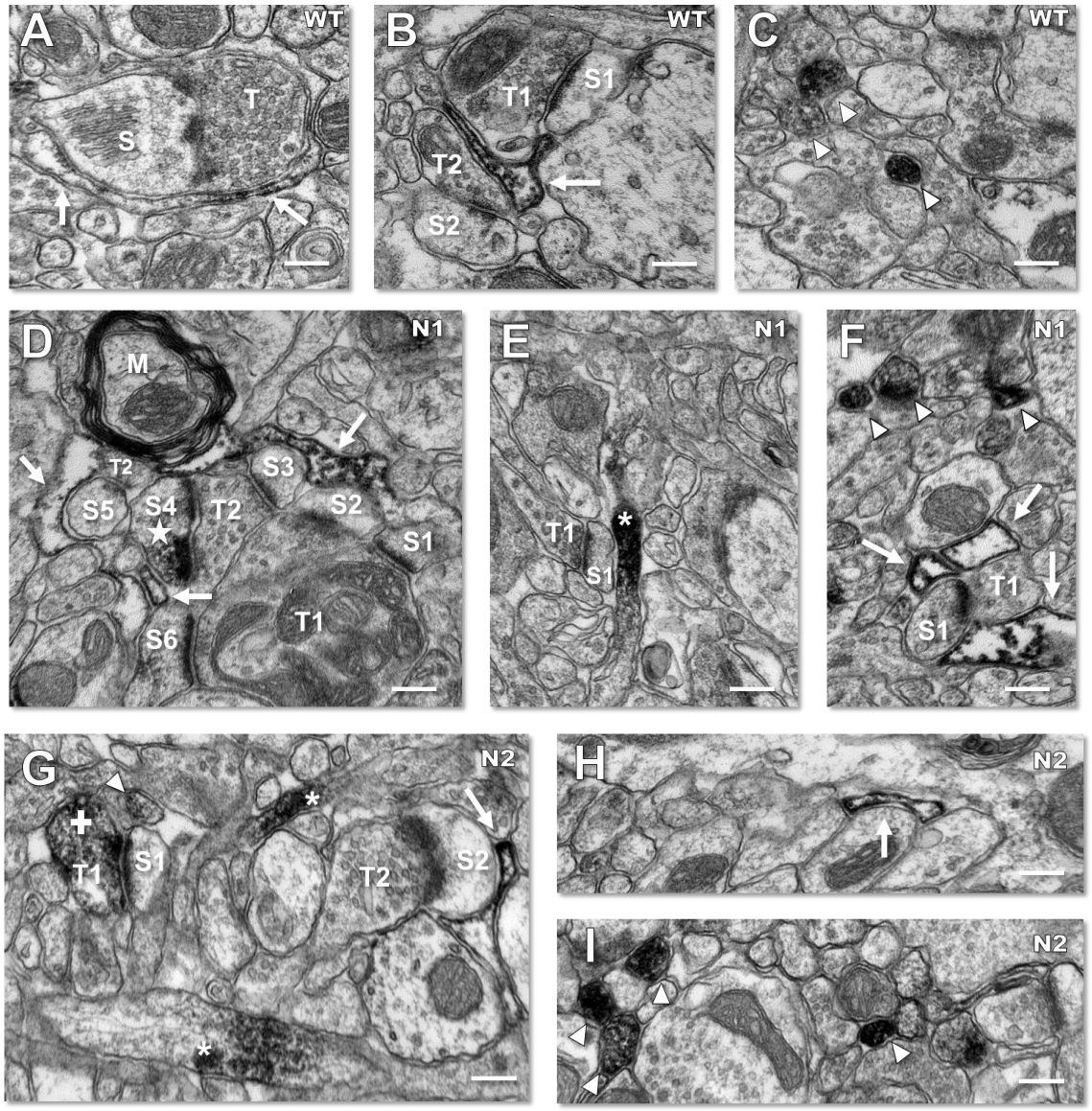


Figure 4.5. Representative images of Group II mGluR labeling in the cortex of wild-type (WT), NHERF-1 (N1) KO, and NHERF-2 (N2) KO mice. A-C) Examples of Group II mGluR labeling in WT mice. **A)** Labeling was often observed in perisynaptic glial processes (arrows) that were positioned near asymmetric axospinous synapses. **B)** Labeled glial processes can also contact multiple synapses; here, one labeled glial process physically contacts two different asymmetric synapses (arrow). **C)** Cross-sections of pre-terminal, unmyelinated axons were also commonly observed, as denoted by arrowheads. However, post-synaptic labeling was rarely

seen. **D-F)** Examples of Group II mGluR labeling in N1 KO mice. **D)** Glial Group II mGluR labeling was also commonly observed. Shown is a labeled glial process (arrow) that surrounds four separate asymmetric axospinous synapses. Interestingly, the labeled glial process also comes in contact with a myelinated axon (M), which is not labeled. Two additional Group II mGluR-labeled processes are present (arrows). **E)** An immunopositive axon cut in parallel passes near an asymmetric axospinous synapse. **F)** Additional examples of labeled axons cut in transverse (arrowheads) and immunopositive glial process (arrows). **G-I)** Increased immunolabeled unmyelinated axons were observed in NHERF-2 KO mice, although the quantification of glial labeling was unchanged from WT and N1 KO mice. **G)** Examples of axonal (arrowheads, transverse; asterisks, parallel), glial (arrow), and axon terminal (cross) labeling. **H)** An example of a labeled glia (arrow) that is not by an asymmetric synapse. **I)** A field of axons is shown in which many are labeled for Group II mGluRs (arrowheads). Scale bar is 0.2 μm. Abbreviations used: T is for terminal, S is for spine.

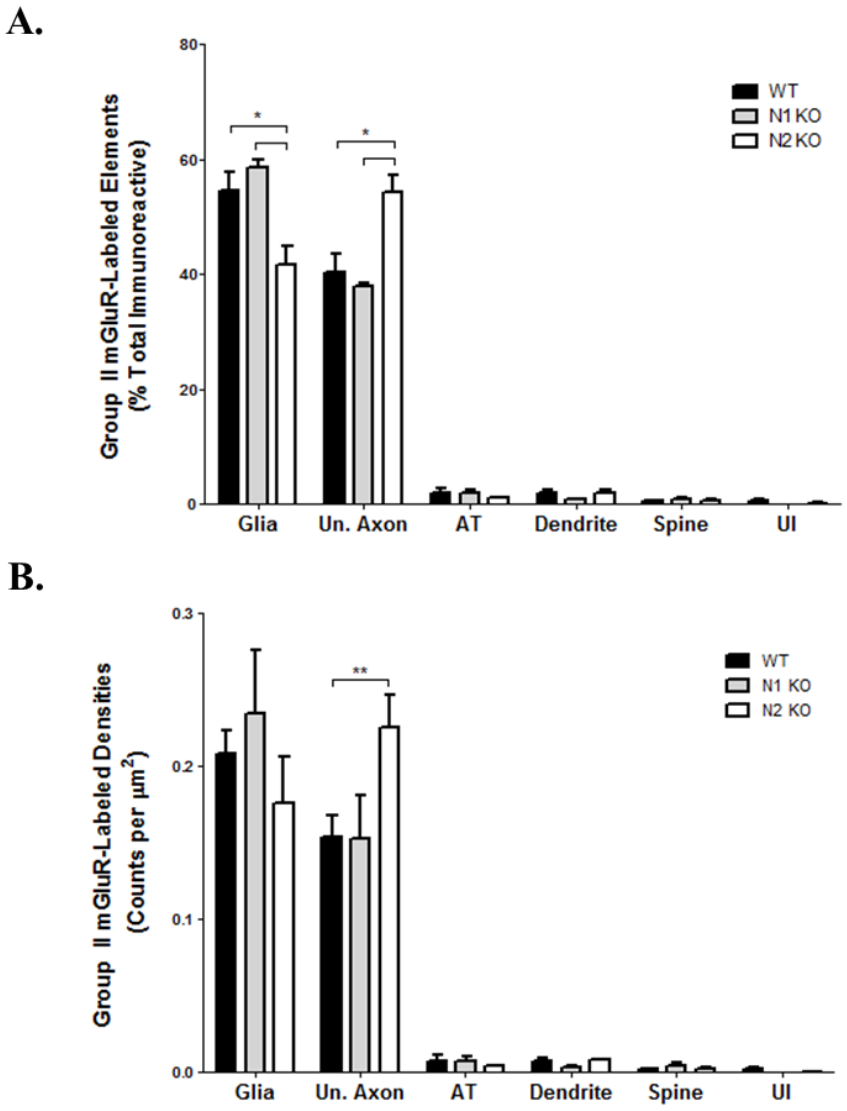


Figure 4.6. Histograms showing the relative distribution or density (counts/area) of Group II mGluR immunoreactive elements in the cortex of wild-type (WT), NHERF-1 KO (N1 KO), or NHERF-2 KO (N2 KO) mice. A) The relative distribution of Group II mGluR labeling was quantified across various elements in the cortex. Data are expressed as percent total of immunoreactive element (mean ± S.E.M.). The predominant elements labeled for all genotypes were glia and pre-terminal, unmyelinated axons (Un. Axon), with infrequent labeling observed in axon terminals (AT), dendrites, and spines. A small fraction of labeling was classified as

unidentified (UI). WT and N1 KO mice had significantly more glia labeling of Group II mGluRs, while NHERF-2 KO mice had significantly more axonal labeling of Group II mGluRs, reflecting a shift in the distribution. (One-way ANOVA Glia: $p = 0.0223$; One-way ANOVA Axons: $p = 0.0238$). **B)** More Group II mGluR immunoreactive axons were counted in N2 KO mice than in WT or N1 KO mice, while similar densities of glial profiles were observed across all three genotypes. Data are expressed as density per μm^2 (mean ± S.E.M.) of labeled elements. A total of 304 (WT), 155 (N1 KO), and 150 (N2 KO) micrographs were examined representing approximately 4496 μm^2 (WT, $n = 6$), 2292 μm^2 (N1 KO, $n = 3$), and 2219 μm^2 (N2 KO, $n = 3$) tissue, respectively. Analysis of Two-Way ANOVA with Bonferonni post-hoc tests for multiple comparisons reveals that N2 KO mice have a higher density of labeled axons in comparison to WT or N1 KO mice (** $p < 0.01$).

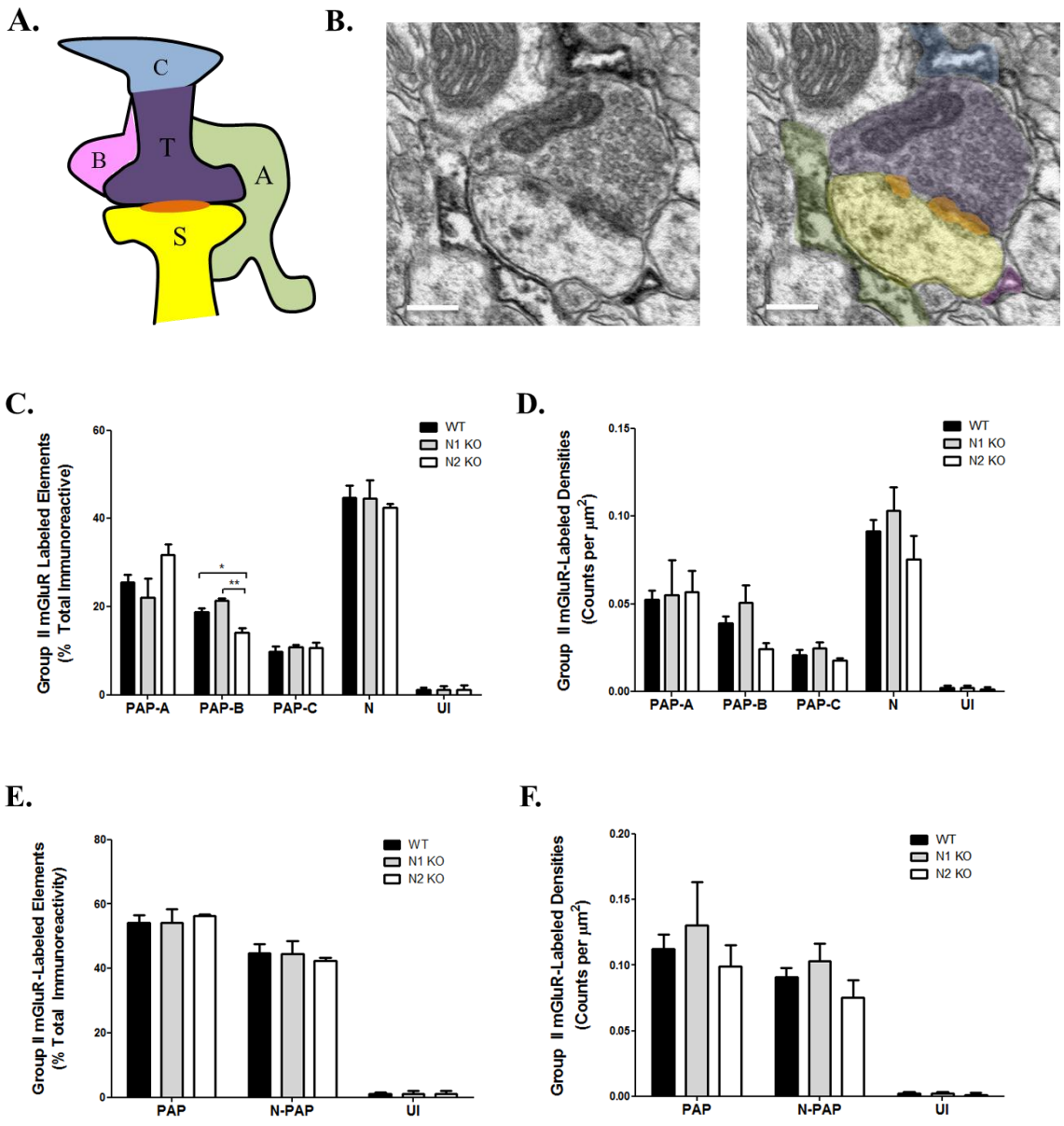
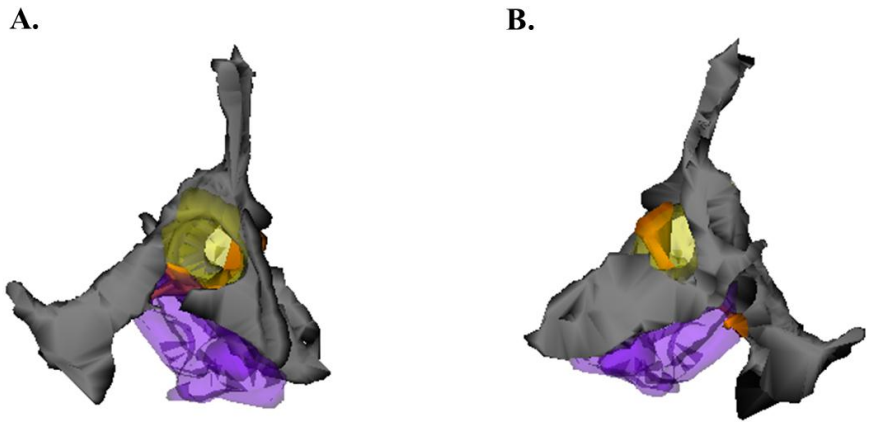


Figure 4.7. Distribution of Group II mGluR-immunoreactivy in perisynaptic astrocyte processes across WT, N1 KO and N2 KO genotypes. A) Group II mGluR-immunopositive astrocyte compartments were subdivided into perisynaptic classifications based on their coverage of the synapse. **B)** Electron micrographs showing the identified elements in accordance with the color classification in **(A)**. One example of each PAP sub-type is shown. **C)** Relative distribution of Group II mGluR-immunoreactivity across perisynaptic astrocyte processes (PAP). Data are

expressed as percent total of immunoreactive element (mean ± S.E.M.).One-way ANOVA tests with Tukey post-hoc tests for multiple comparisons reveal that NHERF-2 KO mice have significantly less Group II mGluR-labeled PAP-B relative to WT or N1 KO mice ($p < 0.0036$). **D)** Data are expressed as density per μm^2 (mean ± S.E.M.) of labeled elements. A total of 304 (WT), 155 (N1 KO), and 150 (N2 KO) micrographs were examined representing approximately 4496 μm^2 (WT, n = 6), 2292 μm^2 (N1 KO, n = 3), and 2219 μm^2 (N2 KO, n = 3) tissue, respectively. No significant differences in PAP or non-PAP densities were observed across genotypes (Two-way ANOVA, post hoc Bonferonni test for multiple comparisons). **E)** Collapse of PAP-A, B, C relative distribution shown in **(D)**. No significant differences were observed across genotypes, although more Group II mGluR labeling was consistently observed across all genotypes, relative to non-PAP or unidentified (UI) labeling. **F)** Collapse of PAP-A, B, C mean densities shown in **(E)**. No significant differences were observed across genotypes. Moderate variation in densities indicates that valid assessments of proportion of Group II mGluR-labeled PAPs versus non-PAPs cannot be made.



Gray = labeled glia, Yellow = Spine, Purple = Terminal, PSD = orange

Figure 4.8. Three-dimensional reconstruction of labeled perisynaptic glial processes: Validation of criteria used in perisynaptic glial classification. Approximately 15 ultra-thin serial sections of Group II mGluR-labeled glia that were participating in a tripartite synapse were used to reconstruct an asymmetric synapse and any surrounding glial processes.

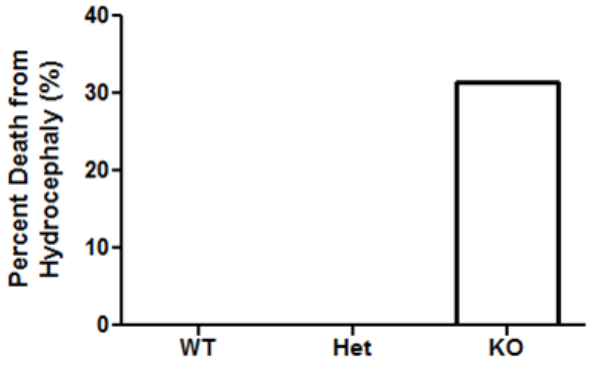


Figure 4.9. Comparison of the percentage of mice that died prematurely from hydrocephaly. Percentage of male NHERF-1 wild-type (WT), heterozygous (Het), and knock-out (KO) mice who survived, as measured up to a 3.5 month time period. Hydrocephaly phenotype was denoted by a swollen head and appearance of sunken eyes, and was initially confirmed via consultations with vet staff. Out of 19 NHERF-1 KO mice that were genotyped, 6 mice developed severe hydrocephaly and either died or were euthanized, while hydrocephaly was not observed in WT (19 out 19) or Het (45 out 45) mice. The phenotype generally presented between the first and second months of age. Those mice whose death was unclear were not included in this analysis. Hydrocephaly was also observed in female NHERF-1 KO mice, although due to the small sample number this was not quantified.

4.4. Discussion

The data presented in this chapter provide the first CNS-specific characterization of the NHERF-1 and NHERF-2 deficient mice. Analysis of the protein expression of NHERF proteins in brain tissue lysates revealed that NHERF-1 and NHERF-2 KO mice do not express full-length NHERF-1 or NHERF-2 proteins, respectively, demonstrating that the design of the knock-out mice was sufficient to disrupt the expression of the full-length NHERF proteins. However, an alternative splice variant of NHERF-2 that encodes for a NHERF-2 protein that lacks PDZ domain 2 was upregulated in NHERF-2 KO mice, relative to WT or NHERF-1 KO mice. The function of this NHERF-2 splice variant is unknown, and despite its upregulation, the expression of the variant in the NHERF-2 KO brain tissue was much lower per unit protein than the expression of full-length NHERF-2 in the WT brain tissue. Thus, the NHERF-2 KO mice clearly lack full-length NHERF-2, but nonetheless all studies performed with these mice should be interpreted with the caveat that they do still exhibit low expression of a putative NHERF-2 splice variant.

To date, three alternative splice variants of mouse NHERF-2 have been conclusively identified. The original mouse NHERF-2 mRNA transcript contains 2118 nucleotides (human 2194 bp) which comprise 6 exons (mouse NM_023055.2; human NM_001130012.2) and encode a 337 amino acid protein, with a predicted molecular weight of 37.5 kDa (mouse NP_075542.2; human NP_001123484.1). In contrast, another reported mRNA transcript of NHERF-2 contains just 1688 nucleotides (mouse NM_023449.3) or 1811 nucleotides (human NM_001252073.1), lacks exon 1, and possesses an ectopic transcription initiation site within intron 1. This splice variant encodes a protein that contains just 226 amino acids (mouse NP_075938.2; human NP_001239002.1), lacks PDZ domain 1, and has a predicted molecular weight of 24.9 kDa. Additionally, another NHERF-2 transcript has been reported by the Georgescu lab in which an expressed sequence tag library (EST) database was searched for NHERF-2 splice variants

(Morales et al., 2004). This splice variant contains 2052 nucleotides and encodes a 317 amino acid protein and lacks exon 6, which encodes residues 264-286 and represents part of the linker region between PDZ domain 2 and the ERM binding domain located at the extreme C-terminus (EST Genbank accession number BC070458, mouse). A few additional NHERF-2 splice variants have been identified at the transcript level in the human and recently published to Genbank. Most of these variants also lack a PDZ domain, similar to the mouse and human transcript variant that encodes just 226 amino acids.

The function and localization of the alternative transcript variants of NHERF-2 remains unknown and will be worth future investigations. For the NHERF-2 transcript variant that lacks the first PDZ domain, it will be intriguing to discern to what extent this protein functionally overlaps with full-length NHERF-2. In the case of the Homer scaffolding proteins, which like the NHERF proteins can associate with mGluRs, Homer1a represents a splice variant with a shortened C-terminus that lacks the coiled-coiled (CC) domain required for self-association; this splice variant can act as a dominant negative on the remaining Homers that have longer C-termini and possess the CC domain (Tu et al., 1998). Likewise, the NHERF proteins have been shown to self –associate and form homo- and hetero-oligomers with each other via the association of their PDZ domains (Lau and Hall, 2001). Therefore, one could conceive that if a PDZ domain was lacking, that NHERF protein would be unable to simultaneously bind its PDZ-ligand and a respective NHERF protein, thereby greatly diminishing the likelihood of the formation of multimolecular signaling complexes. Moreover, this shortened PDZ protein may too have a dominant negative function, similar to Homer1a.

We did not observe any measurable difference in the total expression of either GLAST or Group II mGluRs across cortex, striatum, hippocampus, and cerebellum (Group II mGluRs only) from NHERF-1 or NHERF-2 KO mice, relative to WT mice. We had hypothesized that loss of NHERF-2, and possibly NHERF-1 as well, would lead to a reduction in the total expression of

GLAST, based on the *in vitro* biochemical experiments shown in Chapter 2 in which NHERF-2 was shown to stabilize GLAST expression in cultured astrocytes. The similarity in GLAST expression levels between WT and NHERF KO brain tissue may reflect compensatory differences that occur as a result of NHERF-2 being absent throughout development, whereas the *in vitro* approach examined GLAST expression following transient knock-down of NHERF-2. Additionally, it is possible that the expression of the putative NHERF-2 splice variant in the NHERF-2 KO mice might be sufficient to functionally compensate for the loss of full-length NHERF-2. Similarly, it is possible that both NHERF-1 and NHERF-2 are able to functionally compensate for the loss of the other, such that deficits in the glutamate signaling system *in vivo* can only be observed when expression of both NHERF proteins is lost. To test these ideas, it would be necessary to assess GLAST and mGluR expression levels *in vivo* in KO mice in which NHERF-1 and NHERF-2 can be conditionally and fully knocked out, as such mice would avoid developmental compensation issues and also probably exhibit greatly enhanced survival relative to the non-conditional NHERF-1/-2 double KO mice.

Recent work has shown that Group II mGluRs and mGluR5 can be detected in glial processes that ensheath asymmetric synapses in the hippocampus of rodent brain, a particular compartment of astrocytes termed the perisynaptic astrocyte process, or PAP (Lavialle et al., 2011). Additionally, ezrin, the actin binding protein, has also been shown to be particularly abundant in this perisynaptic compartment and interestingly does not appear to overlap with the intermediate filament protein GFAP (Derouiche et al., 2002; Derouiche and Frotscher, 2001; Lavialle et al., 2011). Finally, analysis of the relative distribution of NHERF-2 in the rodent brain revealed it is most commonly found in glia, and examples of NHERF-2 labeling in the PAP have been reported, although the proportion of PAP labeling has not been quantified (Paquet et al., 2006b). Given the known localization of Group II mGluRs, mGluR5, ezrin, and NHERF-2 in the PAP and the fact that NHERF-1 and NHERF-2 contain an ezrin binding domain, led to the

hypothesis that NHERF proteins may link their mGluR-interacting partners to ezrin and the actin cytoskeleton, thereby selectively targeting them to PAPs.

To test this hypothesis, we first examined the relative distribution of Group II mGluR immunolabeling across WT, NHERF-1, and NHERF-2 KO mice. For all genotypes examined, Group II mGluR labeling was selectively localized to either glia or unmyelinated axons, with axon terminals and post-synaptic labeling in dendrites and spines much more rarely observed. Although the expression pattern was consistent across genotypes, blinded quantification of the data revealed that WT and NHERF-1 KO mice exhibited more glial Group II mGluR labeling, relative to axonal labeling. In contrast, NHERF-2 KO mice exhibited more axonal labeling, relative to glial Group II mGluR labeling, suggesting either a shift in glial or axonal Group II mGluR distribution when expression of full-length NHERF-2 is lost. Interestingly, no differences in the density of glial Group II mGluR labeling was observed between the genotypes; however, a significant increase in the mean densities of axonal Group II mGluR labeling was observed in the NHERF-2 KO mice, suggesting that the shift in distribution corresponded to an increase in immunoreactive axons in the NHERF-2 KO mice rather than a loss of glial Group II mGluR labeling. Although NHERF-2 has been detected in unmyelinated axons, the role of NHERF-2 in neurons is not known (Paquet et al., 2006b). Future studies should examine how loss of NHERF-2 can lead to the increased axonal targeting of Group II mGluRs.

In addition to categorizing the expression of Group II mGluRs in various neuronal elements and glia, we further subdivided the labeled glial elements in terms of whether they constituted perisynaptic astrocyte processes (PAPs). In this case, only perisynaptic astrocyte processes ensheathing asymmetric synapses were examined. The PAP criterion were further sub-divided into three categories, with PAP-A representing the most stringent category of a labeled glial element that physically touches both the pre- and post-synaptic element of an asymmetric synapse. PAP-B and PAP-C corresponded to a labeled PAP touching either the pre- or post-

synaptic membrane or a PAP distal to the synapse, respectively. Regardless of whether these data are examined as individual PAP categories or if all three PAP categories are collapsed together and compared to non-PAP glial elements, no appreciable differences were observed across genotypes. The lack of any observed differences in Group II mGluR localization to PAPs does not support the hypothesis that NHERF-1 or NHERF-2 are required to localize Group II mGluRs in glial processes, although the same caveats apply here as those that were mentioned above for the analysis of the GLAST expression data. Most trenchantly, it would have been desirable to examine mGluR2/3 expression in tissue from double NHERF-1/-2 KO mice, but unfortunately the double KO mice were not viable and thus could not be included in this analysis.

Overall, these studies regarding the relative distribution of Group II mGluR labeling expand on what is known about the cellular and subcellular distribution of these receptors in the rodent brain. Our anatomical findings are consistent with other reports that demonstrate that Group II mGluR labeling can be observed in pre-terminal unmyelinated axons, glial processes, spines, dendrites, and axon terminals (Muly et al., 2007; Petralia et al., 1996; Sun et al., 2013; Tamaru et al., 2001). Based on mRNA studies in rodents, the Group II mGluR immunoreactivity observed in glia is thought to correspond with mGluR3 labeling of astrocytes and not mGluR2 (Fotuhi et al., 1994; Tanabe et al., 1993; Testa et al., 1994), although one report has found otherwise (Phillips et al., 2000). Recently, the relative distribution of Group II mGluRs in the cortex and hippocampus was examined and found to also predominate in presynaptic elements and glia, with infrequent labeling observed in post-synaptic elements (Sun et al., 2013). However, Sun and colleagues reported enhanced Group II mGluR labeling in axonal elements, rather than glial, which may reflect differences in the area of cortex examined and/or the application of the criteria used to identify elements (Sun et al., 2013). Our studies also expand on these findings by quantifying the proportion of Group II mGluR-immunoreactive elements in the cortex, as we found that Group II mGluR-immunoreactive glia in the WT animals corresponded

to approximately 47% of all glia identified in the mouse cortex. This highlights a significant role of Group II mGluRs in glia, which based on morphological criteria, most likely correspond to astrocytes. As with any quantification of anatomy, technical considerations regarding the antibody penetration, sampling of images used, and application of criteria may influence the outcome of the results. Future studies that endeavor to replicate these results will help to solidify what is known regarding the distribution of Group II mGluRs at the cellular and subcellular level in the cortex.

Lastly, we observed a hydrocephaly phenotype in the NHERF-1 KO mice, which together with the ventriculomegaly, support the notion that NHERF-1 KO mice are more susceptible to hydrocephaly, than WT littermate controls. As shown in Figure 4.8, approximately 32% of male NHERF-1 KO mice developed severe hydrocephaly that resulted in either premature death or required euthanasia. No hydrocephaly was observed in corresponding WT or Het mice that were born from the observed litters. Additionally, some female NHERF-1 KO mice also had hydrocephaly, but as we primarily used males for our studies, the effect in the females was not quantified. Our observation of the hydrocephaly phenotype is in accordance with the original report of NHERF-1 KO mice from the laboratory of Ed Weinman, from whom we had acquired these mice (Shenolikar et al., 2002). Additionally, we expand on this original report by demonstrating that hydrocephaly was also observed in male mice, and moreover we provide quantification of this phenotype in males. However, it should be pointed out that additional NHERF-1 KO mice have been reported, with no obvious signs of hydrocephaly or survival issues (Morales et al., 2004). Interestingly, these NHERF-1 KO mice are also on background C567BL6/J, but were generated via a retroviral gene trapping method to target exons 1-4 of NHERF-1, whereas the NHERF-1 KO mice utilized in our studies were generated via a neomycin insertion into exon 1 of NHERF-1. Thus, it is possible that the differences in the methods used to disrupt the NHERF-1 gene are meaningful for the phenotype. Moreover, an intriguing

explanation for the presence of the hydrocephaly phenotype may result from the role recently attributed to NHERF-1 in the organization of the primary cilia (Francis et al., 2011), since deficits in motile and/or primary cilia have been shown to lead to hydrocephaly in mouse models (Banizs et al., 2005; Carter et al., 2012; Davis et al., 2007; Friedland-Little et al., 2011; Ibanez-Tallon et al., 2004; Tissir et al., 2010). This phenotype highlights an important role for NHERF-1 in the control of fluid homeostasis in the CNS and should be further explored in the future. It remains to be determined if NHERF-2 deficient mice are susceptible to hydrocephaly. The observed ventriculomegaly is supportive of this notion, but no obvious phenotype was observed. Thus, a more thorough study of NHERF-2 KO mice should be done in order to ascertain if NHERF-2 KO mice are also at risk for developing hydrocephaly.

4.5. Materials and Methods

Animals: All mice used in this study were in accordance with IACUC rules and guidelines at Emory University. Wild-type (WT) mice used for the expression studies were purchased from Jackson Labs (C57BL6/J), while NHERF-1 and NHERF-2 KO mice were housed, water, food ad libitum. Mice used in the expression studies were between adults between 3 to 5 months of age. Mice used for the electron microscopy studies were selected after crossing NHERF-2 mice to NHERF-1 mice and selecting NHERF-1 Het/ NHERF-2 Het breeders to be founders of the colony. NHERF-1 Het or NHERF-2 Het mice were then selected from the F1 generation be the founders for separate NHERF-1 or NHERF-2 colonies, in which Het mice were bred together to generate WT littermates and NHERF-1 or NHERF-2 KO mice.

Genotyping: In accordance with IACUC guidelines, tail clips were taken from mice at P11 to P14 postnatal. Tails were then digested with a protease buffer (Proteinase K 2 mg/ml, SDS, EDTA, NaCl) overnight at 55°C and subsequently mixed with isopropranol to precipitate DNA.

Approximately 500 ng/ml of DNA were used for each PCR cycle. Primers for NHERF-1 were originally developed by Shenolikar et al. 2002 (Shenolikar et al., 2002). They are P1(Common Forward): 5' CTC TGT TTA TTC CCA GAA GGA 3'; P2 (Neomycin Cassette, Mutant): 5' CAA GAA GGC GAT AGA AGG CGA TG 3'; and P3 (WT Reverse): 5' GAG CCA GGT TCT ACC AGA CGG ATA AAC TGG 3.' PCR results were run on a 0.8% agarose gel with ethidium bromide to reveal PCR products. Primer combinations of P1 and P2 yielded a 2400 bp fragment that corresponded with the NHERF-1 mutant allele, while primer combinations of P1 and P3 yielded a 1400 bp fragment that corresponded with the WT NHERF-1 allele. The primers for NHERF-2 were originally developed by the Yun lab (Singh et al., 2009). They are P1 (Common Forward): 5' TTC TAT AAG CCT CCA TTT CCT CT 3'; P2 (WT Reverse): 5' CCC ACC CCC ATC GCT GCT C 3'; and P3 (Mutant Reverse): 5' GCG CCA GTC CTC CGA TTG A 3'. PCR results were run on a 2.0% agarose gel with ethidium bromide to reveal PCR products. Primer combinations of P1 and P2 yielded a 303 bp fragment corresponding with the NHERF-2 mutant allele, while primer combinations of P1 and P3 yielded a 229 bp fragment corresponding to the WT NHERF-2 allele. Negative control reactions in which the DNA was not included were also run to control for contamination.

Euthanasia and Tissue Collection: All mice used in the study were euthanized in accordance with IACUC guidelines. Mice used for the expression studies across WT, NHERF-1 KO, or NHERF-2 KO genotypes were euthanized via CO_2 asphyxiation. Following absence of toe pinch reflex, mice were rapidly decapitated and brains were quickly removed. Samples from prefrontal cortex, striatum, hippocampus and cerebellum were micro-dissected and immediately flash frozen on dry ice and stored at -80°C until needed.

Preparation of Brain Homogenates and Normalization of Protein Concentrations: To create the brain homogenates, snap frozen WT, N1 KO and N2 KO brains were thawed sequentially on ice

in 10 ml of ice-cold Harvest Buffer containing 50 mM NaCl, 20 mM Hepes, 5 mM EDTA, 1 protease inhibitor cocktail tablet (Roche Applied Science, cat. # 04693132001), diluted with dH_2O up to 50 ml, pH 7.4. After thawing, the brains were subjected to 15 strokes on a dounce homogenizer to homogenize tissue and lyse cells. The crude homogenates were centrifuged for 15 minutes at 17,000 RPM (~35,000 g) at 4°C. The supernatants were discarded and the pellets were re-suspended in ice-cold Harvest Buffer, snap frozen in liquid nitrogen, and stored at -80°C until needed. Brain homogenates were thawed on ice and protein assays were performed (B.C.A. Pierce) to determine the protein concentrations of the samples. Samples were then re-suspended in 6x Sample Buffer to achieve a 1x final concentration.

SDS PAGE and Western Analysis: Following robust sonication on ice, samples were loaded onto 4-20% Tris-glycine gels and proteins subjected to SDS-PAGE electrophoresis. Proteins were then transferred to nitrocellulose (25 V for 2 hours, at RT). Protein transfer was visualized with Ponceau stain and then the membrane was blocked for 30 minutes in blot buffer containing 2% non-fat milk and 0.1% Tween-20 in dH_2O. Primaries used for expression studies were: rabbit polyclonal anti-mGluR2/3 (Chemicon, catalog # 06-676), guinea pig polyclonal GLAST (Chemicon, catalog # AB 1782, lot LV 1596444 GP, note no longer available), rabbit polyclonal anti-NHERF-1 (Ab 5199, generously provided by C. Chris Yun), rabbit polyclonal anti-NHERF-2 (Ab 2750, generously provided by C. Chris Yun), and rabbit monoclonal anti-actin (Sigma, catalog # A2066). Following 1 hour incubation at RT, membranes were washed for 3 x 5 minutes and then incubated with the corresponding HRP-conjugated secondary (GE Healthcare, ECL™ anti-mouse or rabbit IgG, HRP-linked, whole antibody; or Santa Cruz Biotechnology, goat anti-guinea pig IgG-HRP) for 30 minutes. The membrane was then washed 3 x 10 minutes and rinsed once with deionized water. Bands were visualized using an enhanced chemiluminescence kit (Pierce) and exposed to films for various time-points. The membrane was then stripped with

Restore Buffer (Pierce) and probed with rabbit anti-actin (Sigma) as an internal control for protein loading.

Perfusions: A total of 12 mice were perfused for the electron microscopy experiments. Mice were acclimated for 2 hours prior to being deeply anesthetized with ketamine (between 0.1 and 0.15 ml per mouse) via intraperiotoneal injection. After checking for the absence of a toe-pinch reaction, mice were then transcardially perfused with ice-cold Ringer's solution, followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1 M; pH 7.4, freshly prepared) at a slow, steady rate. After perfusion, mice brains were carefully removed from the skull and immediately transferred to a 4% paraformaldehyde solution to post-fix overnight. Additionally, a sample of tail was taken to confirm genotyping. Brains were then serially sectioned into 60-μm-thick sections using a vibrating microtome and stored in phosphate-buffered saline (PBS) at 4°C until needed.

Primary Antibodies: The primary antibody used was raised against the C-terminal epitope of rat mGluR2-NGREVVDSTTSSL. This epitope is highly similar to the C-terminus of mGluR3 and has been used successfully by others to detect rodent Group II mGluR labeling in the brain (Muly et al., 2007; Petralia et al., 1996). The antibody is from Millipore/Chemicon (AB1553, lot LV1825814). The antibody was used at a dilution of 1:100 or 1.0 μg/ml.

LM Immunoreaction:

Tissue sections were chosen from the same region of the cortex across all genotypes, as assessed via their location in the serial sectioning. Sections were then placed in a 1% sodium borohydride solution for 20 minutes and washed extensively with PBS (phosphate-buffered saline) until bubbles had completely dissipated. Sections were incubated in blocking serum (1% normal goat serum, 1% bovine serum albumin, 0.3% triton, diluted in PBS) for one hour at room temperature

and were then incubated with the primary antibody (Ab 1553 anti-mGluR2/3 1:100 dilution in blocking serum) overnight at room temperature. Following removal of the primary antibody solution, sections were rinsed three times with PBS and then incubated with a 1:200 dilution of a biotinylated goat anti-rabbit IgG (Vector) for one hour at room temperature. The sections were then rinsed three times with PBS and then incubated for an additional ninety minutes in the avidin-biotin peroxidase complex (ABC) solution. Sections were then rinsed two times in PBS and then one final time in Tris-Cl. Immediately prior to use, fresh 3,3'-diaminobenzidine (DAB) solution was prepared using (H_2O_2 and Tris-Cl) and then added to the sections to incubate for ten minutes to reveal the reaction. Sections were thoroughly rinsed at least five times and then carefully mounted onto gel-coated slides. The next day, they were cover-slipped for imaging and long-term storage. For the control reactions, everything was performed as described, except the primary antibody was pre-absorbed with ten times the amount of the mGluR2-CT peptide (Peptide 2.0) overnight at 4ºC and then added to sections, in order to bind up the ability of the antibody to detect any Group II mGluR labeling and measure background.

EM Reaction: Tissue sections were chosen from the same region of the cortex across all genotypes, as assessed via their location in the serial sectioning. Sections were washed 2x in PBS and then incubated in 1% sodium borohydride. Following extensive washing in PBS, sections were sequentially cryoprotected. Pre-embedding immunoperoxidase labeling was then performed. Sections were incubated in blocking solution (same as in the LM reaction, but with excluding triton) and then incubated in the primary antibody (Ab 1553 anti-mGluR2/3 1:100) for 36 hours at 4°C with gentle shaking. After the incubation, sections were rinsed three times in PBS and then incubated with a 1:200 dilution of a biotinylated goat anti-rabbit IgG (Vector) for one hour at room temperature. The sections were then rinsed three times with PBS and then incubated for an additional ninety minutes in the avidin-biotin peroxidase complex (ABC)

solution. Sections were then rinsed two times in PBS and then one final time in Tris-Cl. Immediately prior to use, fresh 3,3'-diaminobenzidine (DAB) solution was prepared using (H_2O_2 and Tris-Cl) and then added to the sections to incubate for ten minutes to reveal the reaction. Sections were thoroughly rinsed at least five times and then carefully mounted onto gel-coated slides. Sections were then processed for electron microscopy by transferring to phosphate buffer (PB, pH 7.4) for 3 x 5 minutes washes and then subsequent treatment with 1% osmium tetroxide in PB to further fix and stain the tissue (20 minute incubation). Tissue was then sequentially dehydrated, beginning with incubation in 50% ethanol and then switching to 70% ethanol (supplemented with 1% uranyl acetate, filtered) for a thirty-five minute incubation in the dark, followed by incubations in 90% and 100% ethanol solutions. Sections were then treated with propylene oxide and immersed in freshly prepared Durcupan resin, mounted onto slides, and baked overnight to set. Small sections were chosen from similar regions of the cortex across all groups and mounted onto blocks to be processed for ultracutting. Sections were collected and mounted onto Piolo-form-coated copper grids. For the 3D reconstructions, small blocks were made and 15 to 25 serial sections were collected from the ultracut and mounted onto copper grids. All grids were counter-stained with lead citrate for five minutes, prior to imaging.

Image Acquisition and Analysis: For the analysis of the relative distribution of Group II mGluR labeling across elements and for the determination of the density of labeling, approximately 50 micrographs were randomly taken of any labeled elements. Tissue was scanned at 10X magnification and then images were acquired at 40X magnification using a 5.0 sec exposure time. Generally, images were acquired approximately one-two fields of view from the resin-tissue interface allowing for sufficient antibody penetration and ultrastructural integrity, without biasing the section towards too little or too much labeling. This was clearly done for the last cohort of 3 WT and 3 NHERF-2 KO mice, although the criteria were not stringently applied for the first

group as the scorer was primarily following criteria to just take images of any observed labeling. The scorer was then blinded to the images and images were scored for Group II mGluR labeling using morphological criteria established by Peters et al. (Alan Peters, 1991).

Hydrocephaly Phenotype: Of the NHERF-1 KO that were generated, the mice who succumbed to hydrocephaly, as noted by the enlarged, pyramidal-shaped head, swollen eyes, and sometimes a penile prolapse was noted in males. Some of the remaining NHERF-1 KO mice were also used for the electron microscopy experiments, in which enlarged ventricles were clearly noted. Therefore, the degree of penetrance of the hydrocephaly phenotype remains unknown. Additionally, it was also observed in females, but as male mice were used for all studies, fewer female mice were kept and monitored and a reliable quantitative estimate cannot be made.

4.6. Acknowledgements

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CHAPTER 5

CONCLUSIONS AND IMPLICATIONS

5.1. Overview

Glutamatergic neurotransmission is essential to normal central nervous system (CNS) function and its dysregulation is associated with many disease states such as epilepsy, schizophrenia, Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS) and stroke and/or ischemia (Beart and O'Shea, 2007; Maragakis and Rothstein, 2001; Nicoletti et al., 2011; Niswender and Conn, 2010; Sheldon and Robinson, 2007). Additionally, targeting transporter-interacting partners and receptor-interacting partners (Bockaert et al., 2010; Ritter and Hall, 2009) has garnered interest as a novel approach to identifying therapeutic targets, in comparison to the more traditional pharmacological approach which targets transporters with activators and inhibitors or receptors with agonists and antagonists. Given the existence of a Type 1 postsynaptic density protein of 95 kDa, disc large tumor suppressor A, and zona-occludens 1 (PDZ)-interacting motif on the extreme C-termini of the astrocytic glutamate transporter GLAST (EAAT1) and the Group II metabotropic glutamate receptors (mGluRs), mGluR2 and mGluR3, we performed a panoramic screen of these C-termini against a proteomic PDZ array containing 96 distinct Type 1 PDZ domains. To this end, we discovered that the Na+/H+ Exchanger Regulatory Factors, comprising NHERF-1 and NHERF-2, could interact with GLAST and the Group II mGluRs. Our primary conclusions were as follows:

1) Interaction between GLAST and NHERF-2 in cultured astrocytes enhances GLAST stability and functionality.
2) Group II mGluRs interact with NHERF-1 and NHERF-2, and mutation of the mGluR2-CT enhances downstream AKT signaling, but this effect does not seem to be due to disruption of NHERF interactions.
3) Loss of NHERF-2 shifts Group II mGluR distribution in the cortex from predominantly glial and axonal localization in WT and NHERF-1 KO mice, to enhanced axonal labeling in NHERF-2 KO mice.

4) Loss of NHERF-1, and possibly NHERF-2, confers susceptibility to hydrocephaly in mice.

In the following sections, I will expand on these conclusions and place them into a broader context, and also discuss future directions and identify the limitations and challenges associated with interpreting these results. Finally, I will end by summarizing the overall significance of this work.

5.2. GLAST interaction with NHERF-2 enhances GLAST stability

The studies described in Chapter 2 revealed that interaction of GLAST with NHERF-2 in cultured astrocytes enhances its glutamate transporter activity, via stabilization of GLAST expression. Our studies expand on the previously observed findings from Lee et al. and Sullivan et al. that demonstrated that NHERF-1 can enhance GLAST functionality (Lee et al., 2007a; Sullivan et al., 2007). Moreover, since the publication of our work in 2011 (Ritter et al., 2011), another lab has replicated our findings that NHERF-2 can enhance GLAST expression. Sato and colleagues observed that either NHERF-1 or NHERF-2 can enhance GLAST surface expression, albeit in a model system of HEK293T cells overexpressing GLAST and tagged versions of NHERF-1 or NHERF-2 (Sato et al., 2012). This work also identified some potential mechanisms underlying the ability of the NHERF proteins to stabilize GLAST at the plasma membrane. First, Sato and colleagues postulated that NHERF-1 can interact with GLAST to facilitate its export from the endoplasmic reticulum (ER), as evidenced by preferential co-immunoprecipitation of NHERF-1 with populations of GLAST containing immature N-glycans. On the other hand, NHERF-2 preferentially associated with versions of GLAST containing mature N-glycans, and NHERF-2 immunolabeling was found to co-localize with Rab5 (early endocytic marker) and ERGIC53 (ER-golgi intermediate compartment marker), suggesting that NHERF-2 may play a role in early endosome and/or recycling vesicles, as well as the transfer of cargo from the ER to

golgi, respectively (Sato et al., 2012). Thus, it is possible that NHERF interactions with GLAST in the biosynthetic pathway account for its enhanced half-life and plasma membrane surface expression, however a proof of concept experiment still needs to be performed. Alternatively, it remains to be determined if the ERM binding domain on the NHERF proteins is required for enhancement of GLAST plasma membrane expression. A recent study suggested that GRK2 can regulate GLAST surface expression in an ezrin-dependent manner, in which a few mechanisms were hypothesized including that these interactions involve scaffolding of GLAST by NHERF-1, GFAP, and the phosphorylated version of ezrin, which is reduced in GRK2 KO mice (Nijboer et al., 2013).

A number of glutamate transporters have now been shown to be regulated by PDZ proteins. For example, PSD-95 interaction with GLT1b has been shown to enhance GLT1b surface expression at the plasma membrane, by decreasing GLT1b endocytosis (Gonzalez-Gonzalez et al., 2008). Additionally, EAAT4 interaction with GTRAP48 (also known as PDZ-RhoGEF) has been shown to stabilize EAAT4 at the plasma membrane, however this mechanism is not fully understood (Jackson et al., 2001). More recently, it was shown that disruption of the type 1 PDZ-interacting motif on the C-terminus of EAAC1 led to a significant reduction in the ability of EAAC1 to transport aspartate, demonstrating that interaction of EAAC1 with PDZ protein(s) can enhance EAAC1 function (D'Amico et al., 2010). Interestingly, the authors discerned a possible mechanism for this enhancement in function and found that EAAC1 interaction with PDZ proteins were involved in the ER-to-Golgi trafficking of EAAC1, thereby promoting its forward trafficking to the plasma membrane (D'Amico et al., 2010). Using a yeast-two hybrid assay, the authors went on to show that EAAC1 can interact with PDZ domains 1 and 2 of the NHERF-3 protein (PDZK1) and that co-expression of NHERF-3 with EAAC1 in MDCK cells significantly enhanced the aspartate uptake of EAAC1 (D'Amico et al., 2010). This study

supports the notion that PDZ domains, independent of the ERM binding domain, can enhance the forward trafficking and surface stability of various glutamate transporters.

Finally, it has been suggested by Poronnik and colleagues that GLAST and NHERF-2 are not expressed in the same cell types in the brain and thus cannot be physiological interacting partners (Lee et al., 2012; Lee et al., 2007a). Specifically, Poronnik and colleagues have provided evidence that NHERF-1 is present in astrocytes, while NHERF-2 is predominantly localized to endothelial cells, as assessed via light microscopic analysis. However, these results are in stark contrast to the significant proportion (39%) of identified glial processes which were immunopositive for NHERF-2 in electron microscopy studies (EM) from our lab (Paquet et al., 2006b). This discrepancy is most likely due to the fact that the resolution of light microscopic studies is insufficient to distinguish endothelial cells from the perivascular astrocytes that wrap around blood vessels in the CNS. Thus, the EM approach is better-suited for achieving a full understanding of the cellular and subcellular localization of NHERF-2 in the brain. Furthermore, although our cultured astrocytes were immunopositive for NHERF-1, they were also highly enriched in NHERF-2 relative to cultured neurons or brain lysates (Chapter 2), which provides further evidence that NHERF-2 is expressed in astrocytes. One further point of consideration stems from a transcriptome analysis from the young mouse brain which revealed that NHERF-2 mRNAs are more abundant in acutely isolated neurons and myelinating oligodendrocytes, relative to astrocytes (Cahoy et al., 2008). Although the transcriptome study examined the expression in young mouse CNS cells and looked at mRNA and not protein, it does posit an intriguing hypothesis that NHERF-2 might also have a meaningful role in neurons early on in development, which provides an interesting context for the finding that Group II mGluRs are redistributed to pre-terminal, unmyelinated axons in NHERF-2 KO mice.

5.3. Group II mGluR interactions with PDZ proteins, but not necessarily NHERF proteins, regulate receptor-mediated AKT signaling in cultured astrocytes.

The experiments described within Chapter 3 identified the NHERF proteins as Group II mGluR-interacting partners and revealed that these interactions are dependent on the hydrophobicity of the last amino acid of the Group II mGluRs, analogous to the GLAST/NHERF interaction. Despite a well-defined role for the NHERF proteins in regulating the downstream signaling of their receptor-interacting partners (Ardura and Friedman, 2011; Ritter and Hall, 2009) and that disruption of mGluR2-PDZ associations lead to an enhanced signaling response to AKT, studies of astrocytic Group II mGluR signaling in which NHERF expression was reduced by gene knockout and siRNA knockdown techniques revealed that the observed differences in signaling between WT and mutant mGluR2 were most likely not due to interactions with the NHERF proteins. Thus, the possibility remains that additional PDZ interacting partners that were identified in the PDZ proteomic array, but yet not explored in this body of work, may be responsible for the observed effect.

As identified in the PDZ proteomic array in Chapter 3, the candidate Group II mGluR-interacting proteins include the cystic fibrosis transmembrane conductance regulator-associated ligand, (CAL; also known as PIST, GOPC, and FIG), zona occludens-3 (ZO-3), and PDZ domain containing 1 (PDZK1; also known as CAP70, NHERF-3, CLAMP, or PDZD1). Additional mGluR2-selective PDZ proteins that were identified include tyrosine-protein phosphatase non-receptor type 13 (PTPN13), while identified mGluR3-selective PDZ domains include membrane associated guanylate kinase, WW and PDZ domain containing 3, (MAGI-3), Rho GTPase binding protein 1 or rhophillin (Rhpn1; also known as Grbp), and multi-PDZ domain protein 1 (MUPP1). Overall, a loose correlation exists between the intensity of the signal on the array and the validity of the interaction. Thus, identified interactions will be described below in order of signal intensity.

Of the additional identified Group II mGluR-interacting proteins, the associations with CAL are most robust. Interactions between CAL and mGluR1 (Zhang et al., 2008) or CAL and mGluR5a (Cheng et al., 2010) have been reported, most likely reflecting the similarity in the PDZ-interacting motif expressed on Group 1 and Group II mGluRs. Additionally, the expression pattern of mRNA transcripts for CAL (see GOPC) across CNS cell types indicates that it is expressed in all major cell types in the brain, although its expression is somewhat higher in neurons, oligodendrocyte precursor cells (OPCs) and in oligodendrocytes, relative to astrocytes (Cahoy et al., 2008; Heiman et al., 2008). Given its widespread distribution, CAL is likely to be a Group II mGluR-interacting partner and warrants future investigation. ZO-3 is another candidate Group II mGluR-interacting partner. Although an initial study reported relatively low expression of ZO-3 in the brain in comparison to other tissues (Inoko et al., 2003), a more recent analysis of a transcriptome array of astrocytes, neurons, oligodendrocyte precursor cells, and oligodendrocytes shows that ZO-3 is expressed in all of these cell types, although albeit at low levels (Cahoy et al., 2008). Moreover, as a protein that is known to be localized to tight junctions in the periphery, ZO-3 might be particularly meaningful in choroid plexus epithelial cells, as transcriptome analysis of the choroid plexus revealed that it is expressed in this compartment of the CNS (Marques et al., 2011). Finally, PDZK1 (NHERF-3) may also be a Group II mGluR-interacting partner, although like ZO-3, its expression pattern is relatively low in the transcriptome analysis of acutely isolated CNS cell types (Cahoy et al., 2008; Heiman et al., 2008). Moreover, like ZO-3, PDZK1 mRNAs are also expressed in the choroid plexus (Marques et al., 2011). In summary, based on their mRNA and/or protein expression patterns, CAL is most likely to be a Group II mGluR-interacting partner in various CNS cell types, while ZO-3 and PDZK1 may be Group II mGluR-interacting partners in the choroid plexus. However, transcriptome analysis of the choroid plexus revealed that only Grm3 and not Grm2 mRNAs were

detected, suggesting that mGluR3 is a more relevant ZO-3 and/or PDZK1 interacting partner in the choroid plexus (Marques et al., 2011).

Few mGluR2-selective interactions were detected in the PDZ array, in fact PTPN13 was the only protein identified to interact with mGluR2 and not mGluR3. An initial study revealed that PTPN13 mRNAs are expressed in both cultured astrocytes and acutely isolated astrocytes, as well as in myelinating oligodendrocytes, with no significant mRNA expression found in cortical neurons from early post-natal rodents (Cahoy et al., 2008). However, a more comprehensive analysis of PTPN13 expression in the adult mouse brain revealed that besides being expressed in astrocytes and oligodendrocytes, PTPN13 mRNAs are also expressed in certain neuron populations including corticostriatal neurons, corticothalamic neurons, medium spiny neurons, and cortical interneurons (Heiman et al., 2008). Given that mGluR2 is primarily localized in neurons in most brain regions, it will be interesting to discern whether mGluR2 and PTPN13 can associate in certain neuronal populations. It remains to be determined if mGluR2 can associate with PTPN13 in oligodendrocytes.

Of the candidate mGluR3-interacting partners, MAGI-3 and Rhophillin were some of the more prominent interactions, while the signal intensity for the MUPP1 interaction was relatively weak. Comparable levels of mRNA transcripts for MAGI-3 have been observed in astrocytes (cultured and *in vivo*) and neurons, with slightly less expression in OPCs and myelinating oligodendrocytes (Cahoy et al., 2008). This widespread distribution positions MAGI-3 to interact with mGluR3, supporting that this interaction should be more closely examined in the future. Rhophillin is another candidate-interacting partner that is known to be expressed in some of the same cell types as mGluR3. Interestingly, transcriptome analyses of CNS cell types in the adult mouse revealed that Rhophillin transcripts are primarily found in astrocytes and in certain neuron populations, such as cortical projection neurons and in medium spiny neurons (Heiman et al., 2008). Finally, MUPP1 was also identified as a candidate PDZ-interacting partner for mGluR3.

Analysis of the mRNA expression patterns of MUPP1 mRNAs (encoded by the gene Mpdz) revealed that MUPP1 is expressed in neurons, myelinating oligodendrocytes, and astrocytes, with its highest expression being in cultured astrocytes (Cahoy et al., 2008). In summary, all three mGluR3-selective candidate interacting proteins are expressed in similar cell types to mGluR3. Thus, it might be useful to investigate all candidate interactions via co-immunoprecipitation experiments with full length proteins in cells, but prioritize those that were prominent hits on the array.

Lastly, although we did not conclusively identify a functional consequence of the Group II mGluR and NHERF interaction on receptor signaling, our validation of the interaction with of the full-length proteins in cells and the demonstration of the dependency of the interaction on the hydrophobicity of the last amino acid of the receptor(s), demonstrate that the NHERF interactions with mGluR2 and mGluR3 occur in a cellular context and are highly specific. Unlike the Group I mGluRs, which have many identified intracellular binding partners, much less is known about Group II mGluR-interacting partners (Enz, 2012). To date, a handful of Group II mGluR-interacting partners have been identified, primarily via high-throughput approaches that use recombinant proteins (Enz, 2012). Thus, the NHERFs expand on a relatively short list of Group II mGluR-interacting partners including tamalin (Kitano et al., 2002), protein kinase A (Cai et al., 2001; Schaffhauser et al., 2000), GRKs (Iacovelli et al., 2009), beta-arrestins (Iacovelli et al., 2009), RanBPM (Seebahn et al., 2008), PICK1 (Hirbec et al., 2002), GRIP (Hirbec et al., 2002) and protein phosphatase 2C or PP2C (Flajolet et al., 2003).

5.4. Loss of NHERF-2 Alters Distribution of Group II mGluRs in the Cortex

The primary impetus behind the work in Chapter 4 was to characterize the cellular and subcellular distribution of Group II mGluR immunoreactivity in the mouse brain and determine if the distribution might vary across NHERF-deficient genotypes. To this end, we found that Group

II mGluRs are primarily localized within glia and pre-terminal unmyelinated axons in wild-type (WT) and NHERF-1 (N1) KO mice. However, developmental loss of full-length NHERF-2 in NHERF-2 (N2) KO mice led to a modest redistribution of Group II mGluRs in the cortex, in which significantly more axons were immunolabeled for Group II mGluRs. As the function of Group II mGluRs in pre-terminal axons is mainly to act as autoreceptors to control the release of neurotransmitter from the terminal (Schoepp, 2001), it remains an intriguing possibility that developmental loss of NHERF-2 can lead to a change in glutamatergic neurotransmission, thereby leading to a compensatory upregulation of pre-synaptic Group II mGluRs. In support of this hypothesis, it would be interesting to discern whether GLAST expression is reduced in developing astrocytes in the forebrains of NHERF-2 KO mice, as GLAST is more highly expressed in astrocytes in the cerebrum during development and was reduced in astrocyte cultures treated with NHERF-2 siRNA (Chapter 2). Our Western blots of adult brain tissue did not reveal any significant differences in GLAST levels in brain tissue from the NHERF KO mice, but it is possible that there could be developmental differences in GLAST expression and/or localization when NHERF-2 is deleted. If this was the case, the resulting tone of glutamatergic neurotransmission would presumably be dysregulated, since astrocytic glutamate transporter activity is a key mechanism for clearance of synaptic glutamate. Thus, there would be a compensatory drive to upregulate Group II mGluRs on presynaptic neurons, in order to reduce synaptic glutamate concentrations. This is an intriguing hypothesis that could be examined in the future and would further implicate the NHERF proteins in the regulation of glutamate transporter and metabotropic glutamate receptor localization and function.

We also investigated the hypothesis as to whether loss of either NHERF protein would alter the glial localization of Group II mGluRs. Specifically, we hypothesized that fewer perisynaptic astrocyte processes, or PAPs, would be immunolabeled for Group II mGluRs in brain sections from NHERF KO mice. The rationale behind this hypothesis was that the actin binding

protein, ezrin, has been shown to be enriched in PAPs (Derouiche et al., 2002; Derouiche and Frotscher, 2001) and NHERF-2, which contains an ezrin-radixin-moesin (ERM) binding domain, has also been found to be localized in PAPs (Paquet et al., 2006b). Surprisingly, no differences in mGluR2/3 PAP localization were observed across genotypes, even when various PAP criteria were applied. Ideally, we would also have been able to explore a NHERF-1 and NHERF-2 double KO state, as the possibility remains that expression of one NHERF protein might be able to functionally compensate for loss of the other NHERF protein. However, NHERF-1/-2 double KO mice were not viable, at least on the background on which we were working. Moreover, technical challenges exist with identifying PAPs in two-dimensional images, as a high frequency of false negatives is possible, especially when using the most stringent PAP criteria to only score labeled glial processes that physically touch the pre- and post-synaptic elements of an asymmetric synapse. Future studies in which three-dimensional reconstructions of labeled perisynaptic astrocyte processes across groups could help to more conclusively explore this hypothesis. Alternatively, perisynaptic astrocyte processes could be scored from serial sections, such that one could validate that a labeled process is perisynaptic (Ventura and Harris, 1999). Lastly, as highlighted above, the possibility also remains that another PDZ scaffold might be meaningful for scaffolding Group II mGluRs in PAPs. The candidate Group II mGluR-interacting partners that are preferentially expressed in astrocytes include rhopillin, MAGI-3, PTPN13, and to a lesser extent, CAL and MUPP1. Interestingly, PTPN13 also contains an ERM-binding domain, which makes it a particularly intriguing candidate; however, based on the PDZ proteomic array data, PTPN13 appears to be an mGluR2-selective binding partner. Nevertheless, if loss of both NHERF proteins has no effect on Group II mGluR localization in PAPs, these additional astrocytic PDZ proteins could be explored as candidate astrocytic scaffolds controlling mGluR localization.

5.5. Reconciliation of cultured versus *in vivo* astrocytes: A case study on mGluR3

In the experiments presented in Chapter 3, Group II mGluR signaling in cultured astrocytes was extensively studied and found to require mGluR2 and mGluR3 transfection back into astrocytes, since cultured primary or secondary astrocytes lacked detectable expression of Group II mGluRs, as assessed by Western blot analysis, immunocytochemistry, and functional signaling assays (see Appendix, Figure A.2 and Figure A.3). Even when mGluR3 was re-introduced into the cultured astrocytes via transfection, only a small functional response to activate AKT was observed, highlighting the challenges associated with relying on the absence of functional mGluR3 signaling as a corollary for absence of protein. Despite this technical concern, when these data are taken as a whole (Western blot, immunocytochemistry, and signaling data), it becomes apparent that mGluR3 expression was lost in our cultured astrocytes, which is in stark contrast to the significant fraction of glial Group II mGluR immunolabeling (presumably astrocytic) observed in the mouse cortex, in which approximately 47% of all identified glia were immunopositive (Chapter 4).

Of the research groups who have explicitly examined mGluR3 protein expression in cultured astrocytes, all but one of the research groups have reported their inability to detect and/or challenges with detecting mGluR3 protein in cultured astrocytes via Western blot analysis (Aronica et al., 2003; Bruno et al., 1997; Ciccarelli et al., 1997). For example, Bruno and colleagues demonstrated that they could measure immunocytochemical reactivity of Group II mGluRs in mixed mouse neuron and astrocyte cultures; however, when probing 60 µg of cell lysate from mouse or rat astrocyte cultures, no immunoreactivity was observed via Western blot analysis, despite positive detection of brain lysates (see Figure 6, (Bruno et al., 1997). They postulated that Group II mGluR expression levels (and presumably mGluR3) were too low to be detected by Western blot (Bruno et al., 1997). Likewise, probing of 100 µg of cultured rat astrocyte lysate via Western blot analysis only resulted in the detection of mGluR5 and not

mGluR3 (Ciccarelli et al., 1997). Ciccarelli and colleagues also commented that mGluR3 could not be detected in 150 µg of lysate, as measured using two different C-terminal mGluR2/3 antibodies and following immunoprecipitation of any mGluR2/3 that might have been present in their cultures (Ciccarelli et al., 1997). Similarly, Western blot analysis of human astrocyte cell lysates has been shown to be sufficient to detect mGluR5 (30 µg protein sample), while mGluR3 (even 150 µg cell lysate sample) could not be readily detected (Aronica et al., 2003). However, it was recently reported that Group II mGluRs can be detected via Western blot analysis approaches in astrocyte cultures (Durand et al., 2010); and furthermore that treatment with lipopolysaccharide (LPS) can induce protein expression of mGluR3 (Durand et al., 2010). Moreover, the authors provided some immunocytochemistry of mGluR3 labeling in cultured astrocytes (Durand et al., 2010). In future work it will be of interest to determine why the expression of Group II mGluRs in cultured astrocytes can be so variable depending on culturing conditions. In any case, this discrepancy between the robust mGluR3 expression that is always observed in brain astrocytes and the often-undetectable mGluR3 levels in cultured astrocytes highlights a larger concern as to what extent cultured astrocytes capitulate the properties of astrocytes from the brain.

This question has been extensively explored by the laboratory of Ben Barres, in which comparisons were made of the transcriptome expression patterns of astrocytes acutely isolated from the developing rodent CNS versus primary astrocyte cultures grown in the presence/absence of serum. Their analysis revealed that roughly 2100 genes are selectively enriched in cultured astrocytes in comparison to approximately 2800 genes that instead are selectively enriched in acutely isolated astrocytes from transgenic mice in which the enhanced green fluorescent protein (EGFP) is under the control of the S100β promoter, which allows astrocytes to be identified via fluorescent-activated cell sorting (FACS) of (Cahoy et al., 2008). This differential gene expression is solid evidence that cultured astrocytes are not identical to *in vivo* astrocytes (Cahoy et al., 2008). In line with the observations in our studies, of the 2000+ genes identified as being

differentially expressed between isolated astrocytes and cultured astrocytes, comparison of the expression pattern of Grm3 mRNA transcripts in cultured astrocytes prepared in the commonly used method of McCarthy and de Vellis (McCarthy and de Vellis, 1980) versus astrocytes isolated via FACS from various rodent post-natal days (P1, P7, or P17), revealed that expression for Grm3 is 26.3 fold higher (SAM analysis with a FDR threshold < 1%) in acutely isolated astrocytes, relative to cultured astrocytes (see supplemental Table S20) (Cahoy et al., 2008).

Moreover, when the Barres group applied a more sophisticated and demonstrably physiological immunopanning (IP) method to culturing astrocytes, versus the conventional McCarthy and de Vellis approach, IP-astrocytes that were cultured for 7 days *in vitro* (DIV) had a significant decrease (72 fold, $p < 0.05$) in Grm3 transcripts, relative to IP- astrocytes from P1 brains (Foo et al., 2011). Comparisons of transcript expression of IP-astrocytes from P7 brains with IP-astrocytes from P7 brains that were subsequently cultured for 7 days *in vitro* also revealed that Grm3 transcripts were decreased by 100 fold ($p < 0.05$) in the cultured astrocytes (Foo et al., 2011). Taken together, both studies from the Barres laboratory identify Grm3 as a gene that is significantly downregulated during the astrocyte culturing process, even when an arguably more physiologically relevant method (Foo et al., 2011) is used to isolate astrocytes from rodent brains.

One of the predominant hypotheses thought to account for some of the differences between cultured versus *in vivo* astrocytes is that cultured astrocytes are more similar to the population of reactive astrocytes typically present following injury in the CNS, as signified by the enrichment in the expression of glial fibrillary acidic protein (GFAP) in cultured astrocytes (Cahoy et al., 2008). Although GFAP has long been considered a marker of cultured astrocytes (Eng et al., 2000), astrocytes that express GFAP in the CNS are identified as fibrous astrocytes and are localized within the white matter, which is in contrast to protoplasmic astrocytes that express low or barely even detectable levels of GFAP and are found in the gray matter (Bignami et al., 1972; Miller and Raff, 1984). Additional evidence that Grm3 is downregulated in reactive

astrocytes comes from Group II mGluR protein expression and mRNA studies following spinal cord injury. Interestingly, Group II mGluR expression is significantly down-regulated following spinal cord injury, which occurs on post-injury day 7 and remains downregulated through post-injury day 60, as assessed via Western blot analysis of protein extracts (Mills et al. 2001). Subsequent studies found that Grm3 mRNA, yet not Grm2 mRNA, is significantly down-regulated following spinal cord injury (Hu et al. 2002). Although these studies pooled samples from spinal cord tissue and thus multiple cell types, it is well-known that astrocytes become reactive following spinal cord injury, in a process known as gliosis, in which they form a glial scar to simultaneously restrict the spread of pro-death signals released at the site of the injury, but also end up hindering the re-connection of severed axons (Karimi-Abdolrezaee and Billakanti, 2012). Thus, these studies beg the question as to whether the mechanism whereby astrocytes become "reactive" and upregulate GFAP expression is also involved in downregulating Grm3 expression.

Finally, additional evidence that cultured astrocytes are distinct from *in vivo* astrocytes and that cultured astrocytes do not express significant Grm3 mRNAs comes from a similar transcriptome approach that was undertaken by the cancer genome network to identify genes that are differentially expressed across glioblastoma tumor samples. A classification system for glioblastoma was subsequently developed in which tumors can now be classified as proneural, neural, classical and mesenchymal (Verhaak et al., 2010). The authors then compared the transcriptome signatures of these tumors, in light of the transcriptome profile known for cultured astrocytes, acutely isolated astrocytes, neurons, and oligodendrocytes (Cahoy et al., 2008) and found that the neural and classical types of glioblastoma had a molecular signature that was most similar to acutely isolated astrocytes and neurons. Moreover, the expression pattern of GRM3 was found to be significantly increased only in the neural sub-type of tumor, over the other sub-types. In contrast, cultured astrocytes had a molecular signature that was most similar to the

mesenchymal sub-type of glioblastoma, which is marked by an increase in a number of genes related to inflammation (Verhaak et al., 2010). In turn, Verhaak et al. also disclosed that most glioblastoma cell lines are similar to the mesenchymal tumor type, although since this was just reported and not actually presented as data, it is unclear which cell lines had been tested (Verhaak et al., 2010). This provides unequivocal evidence that cultured astrocytes are different than acutely isolated astrocytes, and may be more useful models for testing treatments for mescenchymal GBM.

5.6. NHERF Proteins and Hydrocephaly: A case for cilia dysfunction?

The observed hydrocephaly phenotype in the NHERF-1 KO mice, along with the enlarged ventricles (ventriculomegaly) observed in both the NHERF-1 and NHERF-2 KO mice together implicate the NHERF proteins in the regulation of fluid homeostasis in the brain. Hydrocephaly is characterized by an expansion of the cerebral ventricles, which may result from impaired flow of cerebral spinal fluid (CSF), failure to re-absorb CSF, and/or over-production of CSF; however, many causes of hydrocephaly are idiopathic in nature (Carter et al., 2012). CSF is produced within the choroid plexus, where it circulates throughout the ventricular system including the cerebrum, cerebellum, spinal cord, and subarachnoid space, where it is ultimately absorbed by the arachnoid villi (Brown et al., 2004b). Non-communicating, or "obstructive" hydrocephaly occurs when one or more of the aqueducts or canals linking the ventricles are blocked, whereas communicating hydrocephaly refers to a build-up of CSF after it has exited the ventricles (NINDS, 2008). It is unknown whether the hydrocephaly phenotype in the NHERF-1 KO mice coincides with communicating or non-communicating hydrocephaly. Future studies could examine if the flow of the CSF is blocked in these mice, as evidenced by a failure of a dye that has been injected into the lateral ventricles to circulate throughout the ventricular system.

It remains to be determined how loss of either NHERF protein could lead to a hydrocephaly phenotype, and more specifically, how loss of NHERF-1 over NHERF-2 may lead to a greater risk for the development of hydrocephaly. A significant amount of work has implicated defective motile (also known as secondary) cilia in the etiology of communicating hydrocephaly, as hydrocephaly is observed in many mouse models of ciliopathies (Banizs et al., 2005; Davis et al., 2007; Ibanez-Tallon et al., 2004; Lechtreck et al., 2008; Tissir et al., 2010). In the CNS, motile cilia are found on ependymal cells lining the ventricles and are required for CSF flow throughout the ventricular system. It is easy to conceive how defects in motile cilia structure and functioning could lead to hydrocephaly, as it results from a failure of the motile cilia to properly beat. More recently, non-motile cilia, or primary cilia, have also been implicated in the development of hydrocephaly (Carter et al., 2012; Friedland-Little et al., 2011). Primary cilia are found on nearly every single cell within the nervous system (Louvi and Grove, 2011). Mechanistically speaking, primary cilia on choroid plexus epithelial (CPe) cells have been shown to be instrumental in the transcytosis of CSF across the cell and into the ventricle (Narita et al., 2010).

There is evidence that the NHERF proteins may be localized to ciliated cells within the choroid plexus. An examination of the choroid plexus dissected from the lateral ventricles in adult rats revealed that NHERF-1 immunolabeling is detected in the ventricular epithelium, as well as at the apical surface of the choroid plexus (Lee et al., 2012). On the other hand, NHERF-2 immunolabeling was observed in the endothelial cells of the choroid plexus (Lee et al., 2012). However, transcriptome profiling of the choroid plexus isolated from the adult mouse supports that both NHERF-1 and NHERF-2 are expressed, as evidenced by a 9.4 fold and 7.8 fold expression in mRNAs, respectively (Marques et al., 2011). The authors stated that the majority of these cells were CPe cells, although it is possible that they also included endothelial cells and pericytes (Marques et al., 2011). Overall, these data implicate NHERF-1, more so than NHERF-

2, in the development of hydrocephaly resulting from defects in the primary and/or motile cilia lining the lateral ventricles. Accordingly, the hydrocephaly phenotype was more pronounced in the NHERF-1 KO mice, rather than the NHERF-2 KO mice. Interestingly, it was recently demonstrated that NHERF-1 acts as a retention mechanism to restrict its interacting partners outside of the basement membrane of the primary cilium, via interactions of its ERM domain with the actin cytoskeleton (Francis et al., 2011). As NHERF-2 has the same ERM binding domain as NHERF-1, it is unknown whether NHERF-2 can also act as a retention barrier and thus impact the organization of proteins found within the primary cilium. Moreover, for both NHERF proteins, it will be interesting to discern whether this function of a retention barrier can also extend to impact the formation of motile cilia. These hypotheses could be explored further and would represent an exciting area of research linking NHERF proteins to the control of fluid homeostasis in the CNS.

Lastly, given the significant amount of work in this dissertation describing the regulation of glutamate transporter and receptor function by the NHERF scaffolding proteins, it is important to speculate whether disruptions in glutamatergic neurotransmission and signaling are known to be involved in hydrocephaly. As revealed by knockout studies, loss of astrocytic glutamate transporters and Group II mGluRs is not linked with an increase in the incidence of hydrocephaly (Aiba et al., 1994; Chiamulera et al., 2001; Linden et al., 2002; Masu et al., 1995; Peghini et al., 1997; Rothstein et al., 1996; Sansig et al., 2001; Takayasu et al., 2005; Tanaka et al., 1997). However, alterations in glutamatergic function have been associated with the effects of hydrocephaly. For example, GLAST mRNA has found to be upregulated in periventricular astrocytes in the acute phase of a kaolin-induced model of hydrocephaly in mice, however even the authors conjectured that this increase in GLAST mRNA most likely corresponded to reactive astrocytes (Masago et al., 1996). Additionally, another hypothesis that links the glutamatergic system with hydrocephaly arises from the relationships between glutamate transporters and

extracellular sodium concentrations. For example, glutamate transporters are driven by the sodium gradient and are thus tightly coupled to sodium potassium ATPase activity; increased glutamate uptake leads to increased activation of the sodium potassium ATPase (Pellerin and Magistretti, 1997). More recently, it has been determined that glutamate transporters and the sodium potassium ATPase can even exist in macromolecular complexes (Rose et al., 2009). The sodium potassium ATPase is one of many sodium transporters that are involved in regulating the ionic composition of the cerebral spinal fluid (Brown et al., 2004b). Besides a well-known link between hydrocephaly and alterations in CSF composition (Del Bigio, 1989), recently it was shown that two subunits of the sodium potassium ATPase are linked to three aspects of brain ventricle development in zebrafish, including the formation and barrier properties of the neuroepithelium lining the ventricles and CSF production, ultimately having implications for the etiology of hydrocephaly (Chang et al., 2012). Thus, it remains to be determined if a dysregulation in sodium-dependent glutamate transporter function exists in the NHERF KO mice, which in turn might alter sodium potassium ATPase function and subsequent ventricular development.

5.7. Cross Talk between Astrocytic Glutamate Transporters and Glutamate Receptors: A Role for NHERF Proteins?

A broader theme that has emerged from this work is to what extent astrocytic glutamate transporters and metabotropic glutamate receptors can function as meaningful interacting partners, given that they are anatomically well-positioned to respond to synaptic glutamate levels and that GLAST (Ritter et al., 2011), mGluR5a (Paquet et al., 2006a), and Group II mGluRs (Chapter 3 and 4) can all interact with NHERFs. Initial studies on mGluR/transporter cross-talk stemmed from observations that activation of Group I and/or Group II mGluRs in primary astrocyte cultures can change the expression levels of GLAST and GLT-1. Specifically, treatment

of astrocyte cultures with 100 μM DHPG, a Group I mGluR agonist, reduced GLAST levels, whereas treatment with 100 μM DCG-IV, Group II mGluR agonist, increased GLAST levels (Gegelashvili et al., 2000). However, at concentrations of 3μM and higher DCG-IV can also activate NMDA receptors (Ishida et al., 1993; Venero et al., 2002; Wilsch et al., 1994), and NMDA receptors have also been found to be expressed in some astrocytes (Lee et al., 2010). Follow-up studies used lower concentrations of DCG-IV, as well as Group I and Group II mGluR antagonists, and also examined if GLT-1 levels could be affected by metabotropic glutamate receptor activation. As assessed via Western blot analysis, activation of mGluR5 with DHPG decreased expression of GLAST and GLT-1, which was reversed with co-treatment of MPEP, an mGluR5 inverse agonist. In contrast, despite the lack of detection of mGluR3 protein in astrocyte cultures, treatment of astrocyte cultures with a Group II mGluR agonist, 3 μM DCG-IV, led to a marked increase in both GLAST and GLT-1 expression, which was blocked with co-treatment of a Group II mGluR inverse agonist, EGLU (Aronica et al., 2003). As, EGLU reportedly has no off-target effects on other, non-Group II mGluR glutamate receptors, it is quite likely that the data do indeed reflect activation of low levels of mGluR3 in cultured astrocytes.

Analysis of glutamate transporter expression levels in Grm2 or Grm3 KO mice also suggests *in vivo* regulation of glutamate transporter levels by metabotropic glutamate receptors. Comparison of protein levels of GLAST or GLT-1 in various regions of the hippocampus of WT versus Grm3 KO mice revealed that both GLAST and GLT-1 protein levels were lower in the dentate gyrus and region CA1, whereas no significant differences were observed between WT and Grm2 KO mice (Lyon et al., 2008). These studies expand upon some of the *in vitro* studies just described, and more convincingly support that there is some level of cross-talk between glutamate transporters and metabotropic glutamate receptors. However, it is unclear in this study if loss of mGluR3 directly decreases astrocytic glutamate transporter expression. Cell-type specific

deletions of Grm3 would help to ascertain whether loss of astrocytic and/or neuronal mGluR3 decreases astrocytic glutamate transporter expression.

Based on these observations, we hypothesized that NHERF proteins might facilitate the formation of a complex between glutamate transporters and metabotropic glutamate receptors. Co-immunoprecipitation studies of mGluR5 with WT GLAST or mutant GLASTM542A revealed that mGluR5 and GLAST can associate in a manner that is independent of GLAST binding with the NHERF proteins (Chapter 2). Similar preliminary data was observed for mGluR3 association with WT GLAST and GLASTM542A (data not shown). These data suggest that GLAST and Group I/II mGluRs can form complexes in cells and that these interactions do not depend on their joint association with the NHERF proteins. However, it remains an intriguing possibility that NHERF proteins might modulate associations between GLAST and the mGluRs, which is a possibility that could be explored in the future. Given the difficulty of culturing astrocytes containing functional mGluR3, it might be best to focus studies in cultured astrocytes on GLAST and mGluR5 interactions. Lastly, because mGluR5 glial expression is lost in adult development (Sun et al., 2013), and that GLAST is expressed at its highest levels in cerebral astrocytes during early development, it might be most relevant to study mGluR5/GLAST interactions in the developing CNS.

5.8. The challenges of studying mGluR3 signaling *in vitro* and potential strategies

The studies of mGluR3 signaling in Chapter 3 highlight the challenges associated with measuring receptor-mediated mGluR3 signaling in various cell culture model systems including transfected HEK293T cells, cultured astrocytes, or cultured astrocytes expressing mGluR3. The latter approach did result in a measurable agonist-dependent activation of AKT, although the magnitude of the effect was quite small and likely complicated the interpretation of whether interactions with NHERF proteins could affect mGluR3 signaling. Thus, future work might focus

on increasing the signal to noise ratio of mGluR3 signaling outputs in culture models. Additionally, a broad look at the field of mGluR3 signaling studies highlights that mGluR3 signaling has been somewhat enigmatic for many of the groups who have studied it.

Initial reports of mGluR3 signaling used stably transfected Chinese hamster ovary (CHO) cells expressing rat mGluR3 and found that glutamate-dependent activation of mGluR3 led to an inhibition of the forskolin-mediated adenylyl cyclase response (Tanabe et al., 1993). This inhibition of adenylyl cyclase activity was abolished with pre-treatment with pertussis toxin, implicating mGluR3-coupling to Gαi/o in the underlying effects on cAMP (Tanabe et al., 1993). In subsequent studies, glutamate stimulation of CHO-K1 (modified CHO cells) cells stably expressing rat mGluR3 led to a 50% maximal inhibition of forskolin-mediated cAMP accumulation, as observed with 1 μM, 100 μM or 1 mM application of L-glutamate (Wroblewska et al., 1997). Interestingly, the higher concentrations (100 μM, 1 mM) of glutamate used failed to elicit the approximately 80% inhibition of cAMP accumulation, as initially observed by Tanabe and colleagues (Tanabe et al., 1993). Moreover, Wroblewska and colleagues observed that the mGluR3-stably expressing CHO-K1 cell lines failed to respond to application of glutamate, NAAG, or trans-ACPD (Group I and Group II mGluR agonist, mGluR4 agonist at $\geq$ 100 μM) with progressive passage in culture, such that only the first two passages of mGluR3-stably expressing CHO-K1 cells responded in agonist-mediated inhibition of cAMP accumulation (Wroblewska et al., 1997). These results could not be explained by a failure of the cells to produce mGluR3 mRNAs and similar results were also obtained when mGluR3-stably expressing clonal cell lines were made in human embryonic kidney (HEK293) cells, suggesting a more global concern (Wroblewska et al., 1997). Finally, these issues were not observed in the more closely-related mGluR2-stably expressing CHO-K1 cell lines (Wroblewska et al., 1997), further substantiating the challenges of studying mGluR3 over mGluR2.

The next iteration of experimental design in the study of metabotropic glutamate receptors involved co-expressing the astrocytic glutamate transporter, GLAST, along with either mGluR2 or mGluR3, to arguably control extracellular glutamate levels, analogous to the function of the glutamate transporters in the brain (Schoepp et al., 1997; Schoepp et al., 1996). These studies began with the observation that co-expression of rat GLAST in a Syrian hamster cell line stably expressing human mGluR1α (RGT/mGluR1α), resulted in enhanced coupling of agonist-stimulated mGluR1α to phosphoinositide hydrolysis, presumably by preventing receptor desensitization via GLAST-mediated reduction in extracellular glutamate concentrations (Desai et al., 1995). Specifically, it was found that pre-treatment of the RGT/mGluR1α cells with trans-PDC, a potent, competitive, and non-selective glutamate transporter inhibitor, resulted in the accumulation of extracellular glutamate, as measured by HPLC, in which pre-treatment with 100 or 300 μM trans-PDC, led to the accumulation of approximately 8 μM or 12 μM extracellular glutamate, respectively (Desai et al., 1995). Thus, in the absence of co-expressed glutamate transporters it is possible that the extracellular glutamate concentrations can rise and in turn act on expressed glutamate receptors. Schoepp and colleagues thus capitalized on this approach for studies of human mGluR2 and mGluR3 responses to the Group II mGluR agonists APDC and LY 354740 (Schoepp et al., 1996). However, these cell lines may compound the interpretation of how NHERF proteins might influence Group II mGluR signaling, as GLAST is a NHERF-interacting partner. On the other hand, the cultured astrocyte model used in Chapter 3 also has high GLAST expression, so it could be argued that the RGT cells model astrocytes in that regard. Thus, in order to ask questions about how NHERFs might regulate glutamate receptor signaling, a stably transfected cell line expressing an alternative glutamate transporter, such as GLT-1, could also be used.

Following these initial studies, which were shortly after the receptors were cloned in the mid-1990s, few studies of Group II mGluR signaling in heterologous overexpression systems

were performed. However, in 2009, Iacovelli and colleagues performed studies of human mGluR2 and mGluR3 transiently expressed in HEK293 cells; however, for their cAMP studies they also co-expressed an isoform of adenylyl cyclase V (Iacovelli et al., 2009), which is known to be strongly inhibited by Gαi proteins and intracellular calcium (Sadana and Dessauer, 2009). It is unknown if the expression of the adenylyl cyclase V was required to measure agonist-dependent increases inhibition of adenylyl cyclase, but this may be worth future investigation.

Nevertheless, there is some evidence that Group II mGluR activation can be studied in heterologous expression systems in which receptors couple to ion channels. One of the earliest reports entailed transiently expressing rat mGluR2 or mGluR3 in HEK293 cells stably expressing various subunits of the N-type calcium channel (McCool et al., 1996). With this experimental design, the application of L-glutamate and trans-ACPD resulted in the inhibition of the N-type calcium channel, which was found to be dose and voltage dependent (McCool et al., 1996). Likewise, studies of Group II mGluR regulation of G protein inward rectifying potassium channels (GIRKs) in oocytes were also successful (Sharon et al., 1997).

In summary, additional manipulations have often been required to reliably measure agonist-mediated mGluR3 signaling in heterologous overexpression system. It remains to be determined why the initial approaches from Tanabe and colleagues are no longer commonly used. Moreover, studies in various culture model systems in which mGluRs are endogenously expressed may prove to be quite useful, although the interpretation of these experiments can be challenging because of drug selectivity. For example, no selective agonists exist for mGluR3, independent from NAAG, and thus Group II mGluRs are often studied in concert. Lastly, it is interesting to note that the perfusion set-up appeared to be one of the only approaches that was successful in studying transiently expressed mGluR3. As this issue of extracellular glutamate pertains to the field of ionotropic glutamate receptors as well, it may be worth studying mGluRs in a similar manner. To this end, pre-incubation with antagonists might help to reduce

constitutive activation by glutamate and/or studying cellular responses in a perfusion chamber where there is a constant flow of the extracellular solution may also help to "wash away" extracellular glutamate that is released.

5.9. Concluding Remarks

Glutamate transporters and metabotropic glutamate receptors are integral in the regulation of glutamatergic neurotransmission in the mammalian central nervous system. The conservation of the Type I PDZ-interacting motif on the GLAST and Group II mGluR C-termini imply that PDZ proteins might be critical regulators of glutamatergic transporter and metabotropic glutamate receptor function. By screening these C-termini against a comprehensive PDZ proteomic array, the NHERF proteins were identified as candidate interacting partners of GLAST and Group II mGluRs. In cultured astrocytes, GLAST stability and function were found to be enhanced by interaction with NHERF-2. Moreover, the NHERF proteins were found to be Group II mGluR interacting partners, and although no definitive effects of the NHERF proteins on receptor-mediated signaling were observed, loss of NHERF-2 increased the pre-terminal axonal localization of Group II mGluRs in the mouse cortex. These studies shed light on the regulation of glutamate transporters and receptors by scaffold proteins. Such advances in our understanding of the fundamental biology of the glutamatergic system are critical to the future development of therapeutics targeting glutamate signaling in the brain.

APPENDIX

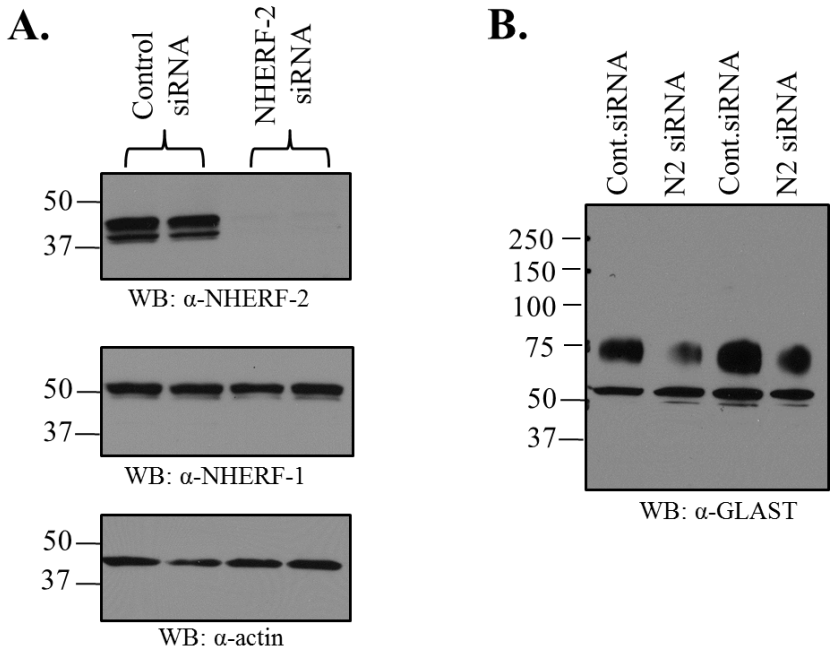

Figure A.1. Treatment of cultured rat astrocytes with NHERF-2 siRNA results in the specific decrease in NHERF-2 expression and the resulting decrease in total GLAST expression. A) Representative immunoblot showing siRNA-mediated knockdown of NHERF-2 expression in rat astrocyte cultures treated with control (1 μM) or NHERF-2 siRNA (1 μM) for 72 hours. Probing of cell lysates with an antibody against NHERF-1 revealed that NHERF-1 levels were unaltered by NHERF-2 siRNA knockdown treatment. Actin serves as a loading control. **B)** Representative immunoblot showing replication of the original finding by Matthew Assay (published in Ritter et al. 2011) that treatment of astrocyte cultures with NHERF-2 siRNA leads to a decrease in GLAST expression. Abbreviations used: N2 siRNA, NHERF-2 siRNA.

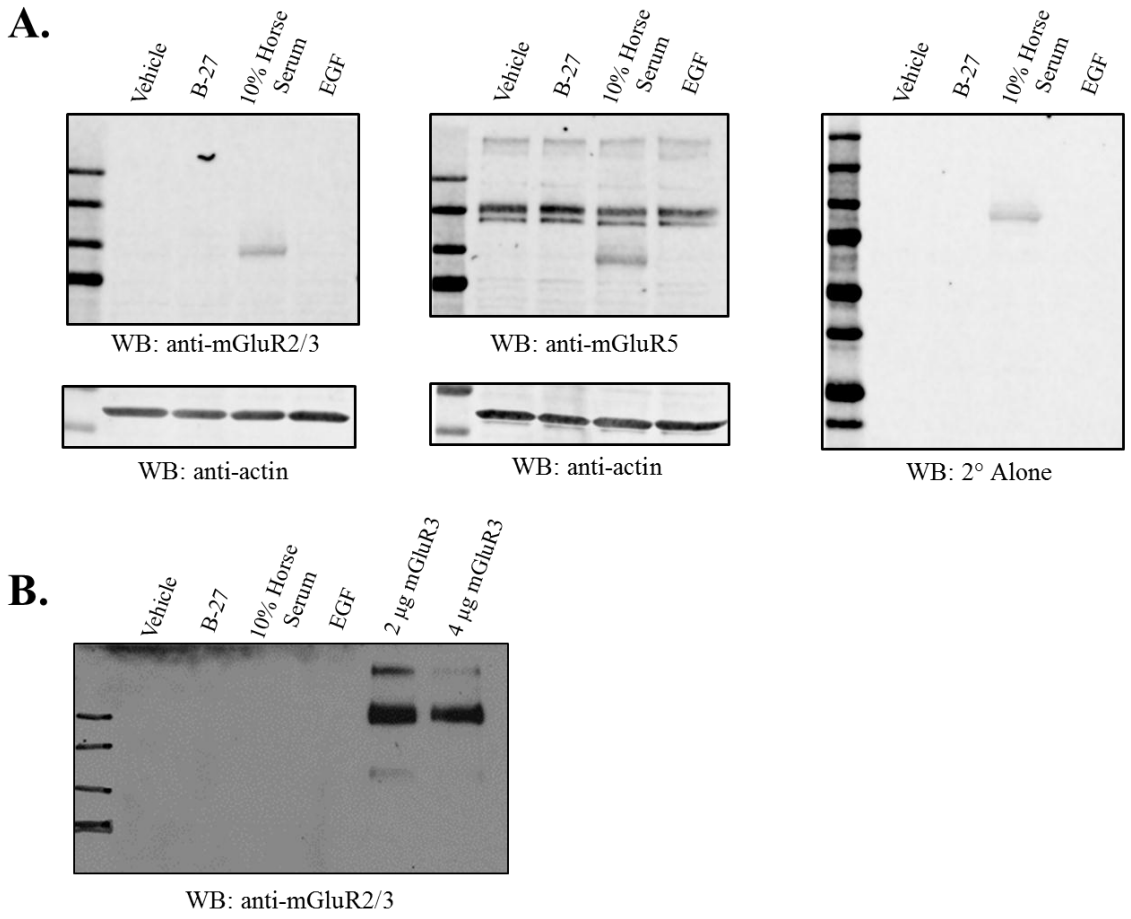

Figure A.2. Group II mGluRs are not detectable in cultured astrocytes via Western blot analysis. A) In accordance with the literature, metabotropic glutmate receptor 5 (mGluR5) is readily detected in cultured astrocytes (middle), while mGluR2/3 is not (left). Secondary alone demonstrates an artifact that is present around 100 kDa in all three blots in **(A)**. Treatment with B-27 supplement, addition of 10% horse serum (in conjunction with 10% fetal bovine serum) or 100 ng/ml of epidermal growth factor (EGF) for 3 days had no effect on mGlu2/3 or mGluR5 expression levels. Actin blots are included as a loading control. **B)** Re-run of the samples in **(A)** plus the inclusion of two positive control of cultured rat astrocytes nucleofected with 2 or 4 µg of mGluR3 cDNAs.

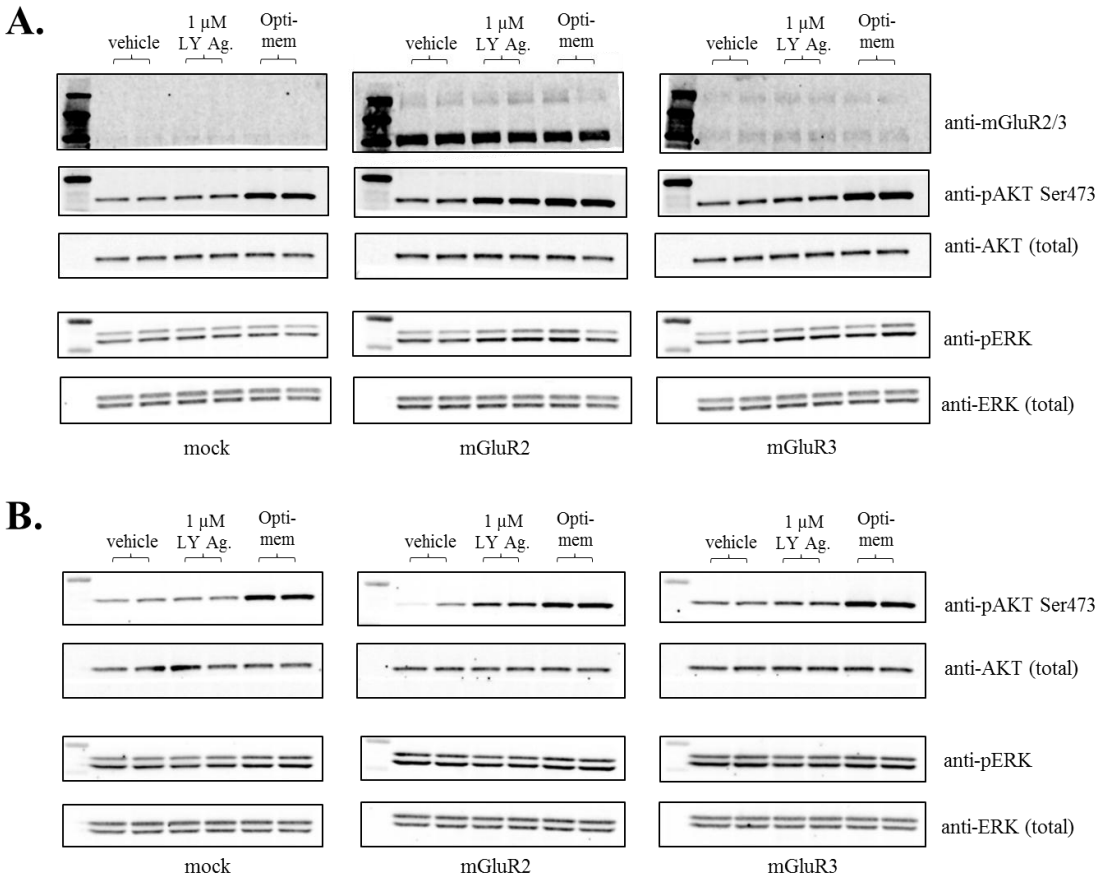

Figure A.3. Preliminary data to ascertain most robust Group II mGluR signaling output in cultured astrocytes expressing mGluR2 or mGluR3. A) Expression of Group II mGluRs in cultured mouse astrocytes led to a measurable increase in phosphorylation of AKT in mGluR2- (middle) and mGluR3- (right) transfected astrocytes, in comparison to mock-transfected astrocytes (left). Note increase in phosphorylation of AKT is more robust for mGluR2, in comparison to mGluR3. A slight receptor-dependent increase in phosphorylation of ERK was observed for mGluR2- and mGluR3-transfected astrocytes. Drugs were applied for 10 minute stimulations. Opti-mem served as the positive control, presumably due to the presence of insulin in the media formulation. **B)** Results from the second experiment replicate initial receptor-mediated AKT effect, but do not replicate the observed increase in phosphorylation of ERK as described in **(A)**.

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