Challenging the Dogma: Reevaluating the Role of Astrocyte Calcium Signaling in Physiology.

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Abstract

Jeremy Christopher Petravic: Challenging the Dogma: Evaluating the Role of Astrocyte Calcium Signaling in Physiology and Behavior.

(Under the direction of Ken. D. McCarthy, Ph.D.)

Gliotransmission represents one of the most important conceptual shifts in neuroscience in the past several decades. Gliotransmission refers to the process whereby glial cells release specific neurotransmitters that modulate synaptic transmission via pre- and postsynaptic activation of G-protein coupled (GPCR) and ionotropic receptors. Astrocytes release glutamate, ATP or D-serine upon activation of a wide range of Gq-GPCRs to modulate neuronal activity in the numerous regions of the brain. These "gliotransmitters" modulate spontaneous and evoked neuronal activity at both excitatory and inhibitory synapses, and affect heterosynaptic depression and long term potentiation in the hippocampus. The theory of astrocyte Ca$^{2+}$-dependent modulation of neuronal activity is built upon the hypothesis that activation of Gq-GPCRs on astrocytes leads to Inositol trisphosphate (IP$_3$) receptor-mediated calcium increases that trigger the release of gliotransmitters. However, evidence supporting this hypothesis has primarily relied on non-physiological methods for increasing or decreasing glial Ca$^{2+}$. For example, uncaging of Ca$^{2+}$ or chelation of intracellular Ca$^{2+}$ in astrocytes affects synaptic transmission. However
the usage of these methods does not specifically target IP₃ receptor-mediated Ca²⁺ increases, considered the primary source of Ca²⁺ changes in astrocytes for gliotransmission.

To probe the role of IP₃ receptors (IP₃R) in Ca²⁺-dependent release of gliotransmitters from astrocytes, we have used the IP₃R type 2 (IP₃R2) knockout mouse model. Through a combination of Ca²⁺ imaging and electrophysiology experiments we found that deletion of IP₃R2 in astrocytes blocks intracellular Ca²⁺ increases in astrocytes in response to Gq GCPR activation. Further, that lack of IP₃R-mediated Ca²⁺ increases does not affect excitatory synaptic transmission of both CA1 and CA3 pyramidal neurons in the hippocampus. Analysis of IP₃R2 conditional knockout mice reveals specific behavioral changes in acoustic startle response and spatial learning in the Morris Water Maze. These novel findings represent a departure from the established theory of gliotransmission and are a significant step forward in our understanding of the role of astrocytic IP₃R-mediated Ca²⁺ increases in physiology.
DEDICATION

To my Mother, Father and my brother Nik, for all your love and support.

To my Mother-In-Law, Joyce Hamel, for all your sacrifice and effort. I wouldn’t have been able to finish without your help.

To my wife Kelly, who keeps me moving forward with her love, kindness, understanding and support.

To my children: Daddy’s finally coming home.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>A/C</td>
<td>associational/commissural</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>APV</td>
<td>(2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate</td>
</tr>
<tr>
<td>ASR</td>
<td>Acoustic startle response</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine trisphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>cKO</td>
<td>conditional knock out</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DHPG</td>
<td>(S)-3,5-Dihydroxyphenylglycine</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>FMRF</td>
<td>Phe-Met-Arg-Phe peptide</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLAST</td>
<td>glutamate-aspartate transporter</td>
</tr>
<tr>
<td>Glt-1</td>
<td>glutamate transporter-1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitrous oxide</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP$_3$R1</td>
<td>inositol 1,4,5-trisphosphate receptor type 1</td>
</tr>
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<td>IP$_3$R2</td>
<td>inositol 1,4,5-trisphosphate receptor type 2</td>
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</tr>
<tr>
<td>KA</td>
<td>kainite</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
</tr>
<tr>
<td>MF</td>
<td>mossy fiber</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>mIPSC</td>
<td>miniature inhibitory postsynaptic current</td>
</tr>
<tr>
<td>MrgA1</td>
<td>Mas-related genes receptor A1</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>OGB-1AM</td>
<td>Oregon Green BAPTA-AM</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendryte progenitor cell</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPI</td>
<td>Pre-pulse inhibition</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SC</td>
<td>Schaffer collateral</td>
</tr>
<tr>
<td>sEPSC</td>
<td>spontaneous excitatory postsynaptic current</td>
</tr>
<tr>
<td>sIPSC</td>
<td>spontaneous inhibitory postsynaptic current</td>
</tr>
<tr>
<td>SR101</td>
<td>sulforhodamine 101</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage gated Ca(^{2+}) channel</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
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Chapter I. General Introduction

1.1. Glial Cells in the Central and Peripheral Nervous System

The study of glial cells in the brain has become a rapidly progressing field of research in neuroscience in the last 30 years. Originally, it was thought that glial cells played a purely supporting role in the brain, providing neurons with metabolic support and maintaining extracellular potassium levels (Somjen, 1988). In the last several decades however, this view of glial cells has changed. Glial cells play an active role in both physiological and pathological processes in the nervous system. Several different subtypes of glial cells have been identified in both the peripheral and central nervous system. These subtypes of glial cells have distinct functional roles in the nervous system, from supporting and maintaining the neuromuscular junction to mediating immune responses in the brain. One of the unifying features that have been identified in nearly all glia subtypes is the use of Ca\(^{2+}\) signaling to interact with their environment. This introduction will present a brief discussion of the various glial subtypes, focusing on their role in nervous system function and involvement in Ca\(^{2+}\) signaling.
1.2. Radial Glia and Ventricular Zone Astrocytes

The primary glial subtype found in the embryonic brain during development is the radial glia. Radial glia provide scaffolding in the developing ventricular zone (VZ) for the migration of excitatory neurons. Radial glias later differentiate into other glial subtypes, primarily astrocytes. Along with glial cells in the neocortical subventricular zone (SVZ), radial glia are also the cells from which the majority of excitatory neurons and some populations of interneurons are produced (Doetsch et al., 1999; Tamamaki et al., 2001; Malatesta et al., 2008). Radial glia-derived neuroblasts from the SVZ migrate to the olfactory bulb using the rostral migratory stream to form inhibitory interneurons (Luskin, 1998).

Calcium signaling is thought to be the primary method by which radial glia communicate with developing neurons to guide their migration. Both radial and SVZ glia express multiple spatial and temporal patterns of Ca\(^{2+}\) activity that rely on intracellular stores. Calcium waves in VZ radial glia are critical for their proliferation and are reliant upon purinergic receptor activation and the phospholipase C-inositol-1,4,5-trisphosphate (PLC-IP\(_3\)) pathway (Weissman et al., 2004). In the SVZ, several different patterns of Ca\(^{2+}\) fluctuations were shown to be present at embryonic stages (Owens and Kriegstein, 1998). Recently it was shown that glutamate released in a Ca\(^{2+}\)-dependent manner from subventricular zone (SVZ) glial cells (primarily astrocyte-like cells) is critical for the survival of neuroblasts entering the rostral migratory stream during their migration to the olfactory bulb (Platel et al., 2010). These data indicate that both during development and in the postnatal brain, Ca\(^{2+}\)
signaling in radial glia and SVZ glial cells is important for the migration of neuronal precursors and formation of the cortex and olfactory bulb.

1.3. Schwann Cells in the Peripheral Nervous System

In the peripheral nervous system (PNS), the primary glial subtype is the Schwann cell. Myelinating Schwann cells ensheathe sections of individual nerve axons in the PNS to promote saltatory conductance along the length of the axon. A single nerve axon is ensheathed by multiple myelinating Schwann cells, but a single Schwann cell only contacts a single axon. In contrast, non-myelinating Schwann cells bundle multiple axon fibers together by surrounding them with their cell bodies to promote axonal maintenance (Griffin and Thompson, 2008). Both of these types of Schwann cells contribute to the development and survival of peripheral neurons.

Schwann cells are especially crucial to the neuromuscular junction (NMJ) where they promote synaptogenesis, regulate synaptic maintenance and potentially modulate NMJ function. Perisynaptic Schwann cells (PSC) at both amphibian and mammalian NMJ can induce synaptogenesis through the NRG-1/ErbB signaling pathway and other unidentified soluble factors (Peng et al., 2003; Reddy et al., 2003; Hayworth et al., 2006). Furthermore, these PSCs are involved in the long term maintenance and reinervation of endplates of adult NMJ (Reynolds and Woolf, 1992; Reddy et al., 2003; Hayworth et al., 2006). PSCs are also involved in modulation of NMJ synaptic function. PSCs will respond to synaptic activity with Ca\(^{2+}\) increases through the Gq-GPCR-PLC-IP\(_3\) pathway to modulate NMJ synaptic activity through
the release of nitrous oxide (NO) or glutamate (Thomas and Robitaille, 2001; Pinard et al., 2003; Rousse and Robitaille, 2006).

PSCs in vivo undergo change to the amplitude and temporal kinetics of their Ca\(^{2+}\) responses in an activity dependent manner (Belair et al., 2010). Blockade of nicotinic receptors (nAChRs) at the NMJ resulted in tuning of PSCs Ca\(^{2+}\) signals that occurred independently of changes to presynaptic release, indicating that PSCs display a form of Ca\(^{2+}\) plasticity (Belair et al., 2010). Immunohistochemical studies indicate that both myelinating and non-myelinating Schwann cells in the PNS highly express IP\(_3\) receptor type 3 (IP\(_3\)R3) and to a lesser degree IP\(_3\) receptor type 2 (IP\(_3\)R2), with inconclusive staining for IP\(_3\) receptor type 1 (IP\(_3\)R1) (Martinez-Gomez and Dent, 2007; Toews et al., 2007). This is in contrast to the central nervous system where IP\(_3\)R3 is primarily found in neuronal cells, and IP\(_3\)R2 is primarily expressed by glial cells (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007). The functional differences in IP\(_3\)R subtypes may play a role in the changes in Ca\(^{2+}\) kinetics observed at PSCs, but further research is needed to determine the causes of the signaling changes.

1.4. Oligodendrocytes in the Central Nervous System

The CNS corollary to the myelinating Schwann cell is the oligodendrocyte, the glial cell responsible for all myelination of nerve axons in the CNS. In contrast to Schwann cells in the PNS, a single oligodendrocyte is capable of contacting and ensheathing multiple neuronal axons. Myelination in the CNS is a highly regulated
process involving multiple pools of oligodendrocyte progenitor cells during
development and is partially modulated by neuronal activity and a specific
differentiation program involving Notch1 and γ-secretase signaling pathways (see
review by Bradl and Lassmann, 2010). Degeneration of the myelin sheath is
associated with the immune disorder Multiple Sclerosis (MS), where abnormally
activated T-cells recognize CNS myelin and initiates a demyelinating inflammatory
response (Kaushansky et al., 2010). During the course of MS, a series of
myelinating and demyelinating phases occur, however the ability to remyelinate
decreases over time for unknown reasons (Piaton et al., 2009). Further research
into the therapies targeting oligodendrocytes may yield future treatments for MS.

Calcium signaling in oligodendrocytes is complex, involving multiple Ca^{2+}
sources. While most of the data concerning Ca^{2+} regulation is originally from culture
studies, there is increasing evidence from in situ studies. Oligodendrocytes express
a wide range of Ca^{2+} permeable channels, as well as mechanisms for release from
internal stores. Ca^{2+} influx across the plasma membrane of oligodendrocytes occurs
by a number of different mechanisms. Oligodendrocytes can sense and respond to
ATP release by neurons, and possibly astrocytes, with Ca^{2+} signals through
activation of either metabotropic P2Y receptors (linked to IP_{3}R based stores) or
ionotropic P2X receptors (allowing Ca^{2+} influx from the extracellular space) (James
and Butt, 2001). Glutamate applied to brain slices also elicits Ca^{2+} signals in
oligodendrocytes, primarily through the activation of Ca^{2+} permeable α-amino-3-
hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainite (KA) and N-methyl-D-
aspartate (NMDA) receptor (Karadottir et al., 2005; Salter and Fern, 2005; Butt,
Oligodendrocytes also release Ca$^{2+}$ from internal stores through both IP$_3$Rs and ryanodine receptors (RyRs), another endoplasmic reticulum (ER) Ca$^{2+}$ release channel. Cultured oligodendrocytes express both IP$_3$R2 and RyR3, and there is extensive functional cross talk between the two Ca$^{2+}$ release channels (Haak et al., 2001).

While Ca$^{2+}$ signaling in adult oligodendrocytes may be important in the maintenance of myelination, in oligodendrocyte precursor cells (OPCs) Ca$^{2+}$ signaling is important for their survival and differentiation (Soliven, 2001). OPCs respond to neurotransmitters with Ca$^{2+}$ increases that can affect their migration, proliferation, gene transcription, development and communication with other cells (Cohen et al., 1996; He et al., 1996; Belachew et al., 2000; Schmidt et al., 2000; Agresti et al., 2005). Similar to mature oligodendrocytes, OPCs can express iGluRs permeable to Ca$^{2+}$, which are involved in OPC migration (Holtzclaw et al., 1995; Deng et al., 2003; Gudz et al., 2006). OPCs also express purinergic receptors linked to both Ca$^{2+}$ influx and release from internal stores. Purinergic signaling in response to neuronal activity is important in the triggering of OPC differentiation into myelinating oligodendrocytes (Stevens et al., 2002; Ishibashi et al., 2006). Based on the literature discussed above, proper Ca$^{2+}$ signaling and regulation by OPCs and mature oligodendrocytes are critical to their development, maturation and proper function in the nervous system.
1.5. NG2 Chondroitin Sulphate Proteoglycan Cells in the Central Nervous System

A recently described subtype of glial cells is the NG2 chondroitin sulfate proteoglycan expressing glial cells, or NG2 cells. NG2 is a membrane proteoglycan found on the surface of several cell types including OPCs, brain capillary endothelial cells and aortic smooth muscles cells (Levine and Nishiyama, 1996). NG2 glia are a population of non-neuronal cells in the brain marked by the expression of NG2 and the alpha platelet-derived growth factor receptor (PDGFRα) (Dawson et al., 2003; Nishiyama et al., 2009). They represent a substantial population (estimated to be between 5-10%) of the brain, and can respond to neuronal activity. Evidence suggests that a proportion of NG2 cells are a separate population of glial cells from oligodendrocytes, microglia and astrocytes in the brain.

Morphologically, NG2 cells were thought to display a similar stellate shape to astrocytes, but further morphological work has refined this view. Whereas astrocytes display several large processes that undergo a series of branches to produce a volume of fine process surrounding the cell body, NG2 cells have a small cell body with small processes that branch earlier and repeatedly (Wigley et al., 2007). Electrophysiologically, NG2 cells express both AMPA receptors and γ-aminobutyric acid (GABA) receptors (Bergles et al., 2000; Lin and Bergles, 2004). Additionally, NG2 cells also possess several types of potassium and sodium currents; however, the exact channels involved and their role in various brain regions is not fully known (Lin and Bergles, 2002; Schools et al., 2003; Chittajallu et al., 2004).
NG2 cells make direct synaptic contact with neurons in the hippocampus and cerebellum, in marked contrast to astrocytes, which typically ensheathe synapses in those regions. For example in the hippocampus, theta-burst stimulation of Schaffer collaterals induces an AMPA receptor-dependent long term potentiation (LTP) of NG2 cell EPSCs (Ge et al., 2006). This long term potentiation of the NG2 cell response is due to increased insertion of Ca$^{2+}$ permeable AMPA receptors at these synapses. In the cerebellum, a single NG2 cell forms multiple synapses with an individual climbing fiber (Lin et al., 2005). This is in contrast to one of the other resident glial types, Bergmann glia, which contact climbing fibers and can sense ectopic release of glutamate at site removed from the nerve terminal (Matsui and Jahr, 2004).

Calcium signaling in NG2 cells has not been fully elucidated. NG2 cells express Ca$^{2+}$ permeable AMPA receptors, which are undoubtedly involved in Ca$^{2+}$-dependent processes in these cells. NG2 cells associated with optic nerves display spontaneous and neuronal evoked Ca$^{2+}$ responses, similar to astrocytes from the same preparation (Hamilton et al., 2009). The same group has also shown that NG2 cells display Ca$^{2+}$ increases through AMPA receptors and purinergic receptors (P2Y$_1$ and P2X$_7$) upon axonal stimulation as well as mechanical stimulation of astrocytes (Hamilton et al., 2010). Calcium signals evoked by activation of P2Y$_1$ but not P2X$_7$ receptors indicates release from intracellular stores via IP$_3$Rs, but to date there are no direct studies of IP$_3$R-mediated calcium release in NG2 cells. This presents an extensive knowledge gap in how NG2 cells might be responding to neuronal activity.
1.6. Microglia in the Central Nervous System

Microglia are not considered “true” glial cells as they are derived from mesodermal tissue whereas all glial cells are derived from ectodermal tissue. Microglia can be found in all regions of the brain and spinal cord and historically has been assigned the function of the brain’s resident immune cell. Microglial cells are involved in injury responses by migrating to the site of insult to act as phagocytes, clearing dying cells and release inflammatory molecules (Streit and Kincaid-Colton, 1995).

Research on the function of resident microglial cells in their inactivated state has been difficult due to limitations of culturing cells or using acute brains slices. Methods used to study microglial cells typically cause them to enter an activated state, in which their expression of ion channels, calcium signaling and secreted molecules undergo a series of changes (Farber and Kettenmann, 2005). Advances in in vivo imaging has allowed the study of resting microglia and given some insight into their potential physiological roles in the brain. For example, it was recently shown using in vivo imaging that the microglial response to injury in the spinal cord can be modulated by ATP and NO (Dibaj et al., 2010). Wake et al. (2009) found that microglia can associate with neuronal synapses to potentially monitor synaptic function and turnover of synaptic connections in ischemia.

One of the hallmarks of microglial cells is that they can enter an activated state in response to changes in their local environment. Upon activation, microglial cells drastically change their gene expression profiles, most notably by increasing
their expression of ion channels. Activated microglia express several ion channels, including inward rectifying potassium channels and chloride channels (Eder, 1998; Boucsein et al., 2000). Microglia can also express a wide variety of neurotransmitter receptors, both ionotropic and metabotropic, as well as cytokine/chemokine receptors (see review by Farber and Kettenmann, 2005). These changes in ion channel and receptor compliment can influence the immune response of microglial cells.

The current literature indicates that microglial Ca\textsuperscript{2+} signaling is mechanistically similar to most glial cells. One of the most prevalent receptor systems expressed by microglia which is connected to Ca\textsuperscript{2+} signaling are purinergic receptors. Microglia express both ionotropic P2X and metabotropic P2Y receptors. Activation of P2Y receptors on microglia are involved in chemotaxis to sites of injury, through activation of Gi/o (Langosch et al., 1994; Honda et al., 2001; Haynes et al., 2006). P2X receptors, particularly P2X\textsubscript{7}, play a major role in the activation of microglia and Ca\textsuperscript{2+} signaling of microglial cells. Activation of P2X\textsubscript{7} receptors leads to depolarization of the microglial membrane and influx of Ca\textsuperscript{2+} from the extracellular space to trigger the Ca\textsuperscript{2+}-dependent release of various substances such as TNF\textalpha and IL-1\beta (Hide et al., 2000; Brough et al., 2002). Additionally, when microglial cells are activated they display a chronic increase in their basal Ca\textsuperscript{2+} levels that impedes receptor based signaling and can modulate the release of substances such as NO and cytokines (Hoffmann et al., 2003). Based on these studies, microglial Ca\textsuperscript{2+} signaling and homeostasis appear to have similarities to other glial subtypes even though microglia are derived from a distinct dermal tissue during development.
1.7. Specialized Glial Cells

Two specialized subtypes of glial cells are the Muller cells of the retina and the Bergmann glia of the cerebellum. Each possesses distinct functional and morphological specializations based on their location, and respond to neuronal activity with Ca^{2+} increases similar to other glial subtypes.

*Muller Cells in the Retina*

Muller cells comprise nearly 90% of the glial cells in the retina and make extensive contacts with retinal neuron subtypes. Muller cells typically have a cell body located in the inner nuclear layer of the retina, with two major processes that extend in either direction into the plexiform layers (Robinson and Dreher, 1990). This morphology allows Muller cells to contact all layers in the retina. It has recently been suggested that this unique morphology allows the Muller cell to act as an *in vivo* correlate of optical fibers, to allow transmission of light through the retinal layers to the photoreceptors (Franze et al., 2007). Muller cells are an important source of trophic factors for the survival of ganglion cells, synaptogenesis and physiology of the retina. Muller cells express and release nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), transforming growth factor β (TGFβ) and numerous other trophic factors (Chakrabarti et al., 1990; Anderson et al., 1995; Seki et al., 2005).

Muller cells express a wide range of neurotransmitter receptors, including but not limited to GABA receptors, ionotropic (iGluR) and metabotropic glutamate
(mGluR) receptors and purinergic receptors (de Melo Reis et al., 2008). The most prominent Ca²⁺ mobilizing receptor system in Muller cells is through several different P2Y family purinergic receptors (Fries et al., 2005). Muller cells can respond to light-induced neuronal activity with Ca²⁺ increases through the neuronal release of ATP (Newman, 2005). This study confirmed pharmacological findings demonstrating that activation of purinergic receptors triggers Ca²⁺ increases and propagation of Ca²⁺ waves in retinal preparations (Newman, 2001). The neuronal activity-induced Ca²⁺ increases were found to be reliant on internal stores. The evidence indicates involvement of IP₃Rs in neuronal activity-dependent Ca²⁺ release in Muller cells since it can be blocked with cyclopiazonic acid (CPA), which depletes internal stores and heparin, which blocks IP₃Rs (Newman and Zahs, 1997; Newman, 2005). Muller cells modulate synaptic activity in the retina through the activity of neurotransmitter transporters. Muller cells abundantly express several types of glutamate and GABA transporters (Biedermann et al., 1994; Eliasof and Jahr, 1996; Lehre et al., 1997). Blockade of glutamate transport influences synaptic activity in the retina by increasing the amplitude and durations of EPSCs (Higgs and Lukasiewicz, 1999).

The physiological relevance of Ca²⁺ signaling in Muller cells is not entirely clear. Muller cells release ATP in response to intracellular Ca²⁺ increases to modulate ganglion cell function through activation of neuronal adenosine receptors. However, no clear link has been established between Ca²⁺ increases and the release of ATP (Newman, 2003). Muller cells express IP₃R2 in the retina, where genetic deletion of this receptor is known to lead to deregulation of osmotic cell swelling, a potential mechanism of ATP release (Wurm et al., 2009). Muller cell Ca²⁺
signaling is involved in retinal vascular control through the arachidonic acid pathway, providing a link between blood flow and neuronal activity through Muller cell Ca\(^{2+}\) stimulation (Metea and Newman, 2006). Further investigation using genetic models and \textit{in vivo} imaging in response to physiological stimulation may provide evidence as to the role of Muller cell Ca\(^{2+}\) signaling in physiology.

\textit{Bergmann Glia in the Cerebellum}

Apart from astrocytes, the cerebellum has a distinct glial subtype called Bergmann glia. Bergmann glia are morphologically distinct from cerebellar astrocytes, having their cell soma in the Purkinje layer with a complex process arbor projecting upwards through the molecular layers toward the cerebellar surface (Grosche et al., 2002). Bergmann glia are related to astrocytes in several ways despite their differing morphologies. Bergmann glia are electrophysiologically related to astrocytes in their membrane properties due to high potassium leak current, linear current-voltage relationship and similar resting membrane potentials (~ -80mV) (Muller et al., 1994; Clark and Barbour, 1997). Bergmann glia are electrically and chemically coupled by connexin gap junctions with neighboring Bergmann glia in a similar fashion to astrocytes (Muller et al., 1996). They are responsible for removal of the excitatory neurotransmitter glutamate from the synaptic cleft of Purkinje cell synapses between climbing and parallel fibers. Bergmann glia express both the glutamate-aspartate transporter (GLAST) and
glutamate transporter-1 (Glt-1) glutamate transporters, with GLAST activity being the most abundant (Rothstein et al., 1994).

Bergmann glia extensively ensheathe both climbing fiber-Purkinje cell (CF-PC) and parallel fiber-Purkinje cell (PF-PC) synapses (Grosche et al., 2002). Bergmann glial cell coverage of these synapses is extensive; Bergmann glia cover close to 100% of CF-PC synapses and nearly 70% of PF-PC synapses in the cerebellum (Xu-Friedman et al., 2001). This morphology, combined with the expression of glutamate transporters, enables Bergmann glia to modulate synaptic function at these synapses by regulating glutamate reuptake (Brockhaus and Deitmer, 2002; Bordey and Sontheimer, 2003; Huang and Bordey, 2004).

Bergmann glia can detect synaptic activity at cerebellar synapses through activation of ionotropic glutamate receptors, primarily Ca^{2+} permeable AMPA receptors (Burnashev et al., 1992). Activation of these receptors causes Ca^{2+} increases in processes of Bergmann glia (Muller et al., 1992). Currently, the physiological roles of these Ca^{2+} increases, particularly in regard to causing feedback onto neuronal activity reported for other glial cells types, is not clear. AMPA receptor mediated Ca^{2+} increases play a critical role in the maintenance of Bergmann glial ensheathment of cerebellar synapses. Elimination of Ca^{2+} permeable AMPA receptors from Bergmann glial leads to retraction of processes, which affects neuronal activity at Purkinje cells synapses (Iino et al., 2001). Calcium increases through these receptors in Bergmann glia regulates the expression and function of glutamate transporters to affect synaptic activity (Lopez-Bayghen et al., 2003; Lopez-Bayghen and Ortega, 2004).
Interestingly, Bergmann glia are targets for ectopic release of glutamate from both climbing fibers and parallel fibers in the cerebellum. While Bergmann glia can sense glutamate release through both glutamate transporters and AMPA receptors, the source of glutamate for these two systems is different. Bergmann glia glutamate transporters respond to spillover from the synaptic cleft with predicted kinetics based on the subtypes expressed (Bergles et al., 1997; Dzubay and Jahr, 1999). In contrast, AMPA receptors on Bergmann glia respond to glutamate released from non-synaptic locations, referred to as ectopic release sites. This was first shown by Matsui and Jahr (2003) who demonstrated that quantal events measured from Bergmann glia and post-synaptic Purkinje cells did not share the same source. Later work by the authors found that glutamate release from synaptic and ectopic release sites induces electrophysiological responses that can be used to differentiate between synaptic and ectopic release sites contacted by Bergmann glia (Matsui and Jahr, 2004; Matsui et al., 2005).

Compared to other glial subtypes, relatively little is known about the function of Gq-GPCR linked Ca\(^{2+}\) release pathways in Bergmann glia. Bergmann glia express a number of neurotransmitter receptors linked to Ca\(^{2+}\) mobilization from internal stores (Kirischuk et al., 1995; Kirischuk et al., 1996; Tuschick et al., 1997; Kirischuk et al., 1999; Singaravelu and Deitmer, 2006). Immunohistochemical data suggests that Bergmann glia express primarily IP\(_3\)R2, similar to other glial types in the brain (Holtzclaw et al., 2002). However, the physiological roles of IP\(_3\)R-mediated Ca\(^{2+}\) signals are not known and remain an open field of investigation.
1.8. Astrocytes in the Central Nervous System

Astrocytes are the predominant cell type in the brain, with the estimated percentage ranging from fifty to eighty percent of the volume of the brain. Astrocytes were historically thought to solely serve a support role in the CNS with their function limited to structural and metabolic support, clearance of neurotransmitters from the synaptic cleft and potassium buffering. In the last 30 years, the concept of astrocytes as solely support cells has evolved as an increasing amount of evidence having been found for astrocytes responding to neuronal activity with intracellular Ca\(^{2+}\) increases (Porter and McCarthy, 1997; Haydon and Carmignoto, 2006). Further, it was discovered that through these Ca\(^{2+}\) increases, astrocytes could actively release compounds to modulate neuronal activity. This represented a significant shift in the study of how synapses operate, and suggested that astrocytes were critical for the proper function of neuronal systems. In this section, there will be a discussion of the support roles performed by astrocytes and an overview of the concept of astrocyte modulation of synaptic activity, in particular the role of Ca\(^{2+}\)-dependent release of neuroactive compounds termed “gliotransmitters”.

1.9. Astrocyte Support Roles in the Central Nervous System

Neurotransmitter Uptake

Astrocytes have long been known to be responsible for the reuptake of neurotransmitters from the synaptic cleft. Astrocytes in culture and in situ express a wide variety of neurotransmitter transporters including but not limited to, glutamate
(Huang and Bergles, 2004), adenosine (Nagai et al., 2005; Peng et al., 2005), dopamine (Karakaya et al., 2007) and GABA (Schousboe and Waagepetersen, 2006). Through the expression of transporters, astrocytes are capable of shaping synaptic transmission through alterations in transporter activity.

The most predominantly studied transporter expressed by astrocytes is the glutamate transporter. Astrocytes express high levels of excitatory amino acid transporters GLAST and GLT-1 (Danbolt, 2001). These transporters are expressed at sufficient density on the processes of astrocytes that even high frequency stimulation of glutamatergic neuronal afferents to release glutamate is not capable of overwhelming the glutamate transporters at most synapses (Diamond and Jahr, 2000). Changes in glutamate transporter activity leads to alterations in synaptic currents, due to prolonged accumulation of glutamate in the synaptic cleft (Barbour et al., 1994; Diamond, 2001). Glutamate transporters also limit the spillover of glutamate to adjacent synapses, thereby reducing synaptic crosstalk at many synapses (Asztely et al., 1997; Min et al., 1998). Expression of glutamate transporters can be regulated by presynaptic elements, providing a novel mechanism by which astrocytes and neurons interact to affect astrocyte function (Yang et al., 2009).

Transport of other neurotransmitters by astrocytes is less well defined than glutamate transport. Adenosine clearance occurs primarily by reuptake by neurons, but astrocytes express the adenosine transporters ENT1 and ENT2 (Nagai et al., 2005). In cultured astrocytes, adenosine transport leads to rapid accumulation of intracellular adenosine, which is then used to synthesize adenine for cellular
metabolism (Peng et al., 2005). Functional expression of the dopamine transporter DAT in striatal astrocyte cultures suggests they may be capable of affecting nigrostriatal transmission (Karakaya et al., 2007). However, cultured cortical astrocytes were found to uptake dopamine not through the dopamine transporter (DAT), but through expression of the norepinephrine transporter (NET) (Takeda et al., 2002), raising the possibility that the mechanisms of dopamine uptake by astrocytes may be brain region-dependent. GABA, the major inhibitory molecule in the central nervous system, is taken up by astrocytes through GABA transporters; however, the functional implications of this transport are not entirely understood (Gadea and Lopez-Colome, 2001; Wu et al., 2006). Recently, a role for astrocyte GABA transporters in modulating the inhibitory tone of the hypothalamic paraventricular nucleus was found (Park et al., 2009). Surprisingly, astrocytes can release GABA through the reversal of GABA transport during glutamate uptake in vivo (Heja et al., 2009). This proposes a novel mechanism for astrocytes to balance excitatory versus inhibitory transmission in the CNS.

Potassium Buffering

One of the most critical functions of astrocytes in the CNS is the uptake of potassium release during neuronal activity. Orkland et al. (1966) was the first to propose that astrocytes act as a potassium sink for extracellular potassium during neuronal activity and shunts the potassium ions through the astrocyte syncytium via gap junctions. The primary potassium channel responsible for this function is Kir4.1,
a weak inwardly rectifying potassium channel (Takumi et al., 1995). This Kir channel is expressed by multiple glial cells types including astrocytes, Muller glia and oligodendrocytes (Butt and Kalsi, 2006; Olsen and Sontheimer, 2008).

Kir4.1 has been implicated in modulating synaptic strength through its potassium buffering function. Cultured astrocytes and spinal cord astrocytes in situ revealed that knockdown of Kir4.1 leads to impairments in buffering capacity and glutamate uptake (Olsen et al., 2006; Kucheryavykh et al., 2007). In agreement with this, Djukic et al. (2007) found that the hippocampal astrocytes of GFAP-Cre conditional Kir4.1 knockout mice had depolarized resting membrane potentials. This reduced membrane potential interfered with both the ability of astrocytes to buffer potassium, as well as their glutamate transporter activity. The impairment of glutamate transport activity can be attributed to the depolarized membrane potential, which would decrease the electrogenic drive required for the active transport of glutamate across the membrane (Tzingounis and Wadiche, 2007). Potassium buffering by astrocytes is therefore important not only in the removal of excess potassium ions from the extracellular space, but necessary to the functioning of glutamate transporters, which in turn can affect synaptic strength.

Cerebrovascular Control

In the last decade, astrocytes were recognized as major mediators of cerebrovascular responses in the brain. Astrocyte endfeet are known to ensheathe the vast majority, if not all, of the vasculature in the mammalian brain (Mathiisen et
In response to neuronal activity, astrocytes can release compounds that cause either constriction or dilation of the blood vessels that they contact (Koehler et al., 2006). This provides a bridge connecting increases in blood flow and increases in metabolic demand during neuronal activity that underlies the hemodynamic response.

While there is abundant evidence that astrocytes are involved in the cerebral vascular response, the exact mechanism of how this response is mediated remains unresolved. Published data implicates both potassium channels and the formation of arachidonic acid metabolites in the vascular response (Zonta et al., 2003; Filosa et al., 2006; Girouard et al., 2010). One unifying feature of astrocyte-mediated regulation of vascular tone is the reliance on astrocytic Ca\textsuperscript{2+} responses. There are two proposed mechanisms for Ca\textsuperscript{2+}-mediated astrocytic control of vascular responses. First, Ca\textsuperscript{2+} elevations in astrocyte endfeet open Ca\textsuperscript{2+} sensitive potassium channels, releasing potassium which activates smooth muscle cells to cause vasoconstriction or dilation (Filosa et al., 2006). However, an argument has been made that potassium siphoning from glial cells is not involved in mediating vasoconstriction (Metea et al., 2007). An alternate mechanism involves increases in intracellular Ca\textsuperscript{2+} that lead to the formation of arachidonic acid metabolites, primarily epoxyeicosatrienoic acid (EETs) and prostaglandins, which induces vasodilation and 20-hydroxyeicosatetraenoic acid (20-HETE) which causes vasoconstriction (Mulligan and MacVicar, 2004; Metea and Newman, 2006; Petzold et al., 2008). The predominant view is that the astrocytes modulate cerebral blood flow through arachidonic acid metabolites rather than potassium release (Koehler et al., 2009).
Several *in vivo* studies support a role for astrocytes in the hemodynamic response. Takano et al (2006) reported that uncaging Ca$^{2+}$ in astrocytes of rodent somatosensory cortex lead to a cyclooxygenase-1 (COX-1)-dependent vasodilation of cerebral arteries. Similar results were found by Zonta et al (2003) upon forepaw stimulation, which were blocked by use of an mGluR antagonist. Astrocyte control of bloodflow has also been shown in the olfactory glomeruli of mice upon odorant stimulation (Petzold et al., 2008). In that study, astrocytes were found to react to presynaptic release of glutamate through activation of mGluRs and COX-1 to induce vasodilation. Interestingly, evidence was also found for a glutamate transporter-dependent mechanism for changes in cerebral blood flow. It was proposed that glutamate uptake may induce small localized changes in Ca$^{2+}$ that could not be detected due to technical limitations. In support of this, a glutamate transporter-dependent mechanism of Ca$^{2+}$ signaling in astrocytes was recently shown in the visual cortex of ferrets in response to visual stimulation *in vivo* (Schummers et al., 2008).

Taken together, *in situ* and *in vivo* studies demonstrate that astrocytes are key regulators in cerebral blood flow. While there remains some debate concerning the role of potassium channels in the control of cerebral blood flow, there is solid evidence for the involvement of arachidonic acid metabolites (Filosa and Blanco, 2007; Iadecola and Nedergaard, 2007; Koehler et al., 2009). Regardless of the mechanism, increases in intracellular Ca$^{2+}$ in astrocytes appear to be a crucial component in the pathway.
**Metabolic Support**

During neuronal activity, neurons consume glucose to produce energy to maintain synaptic transmission (Brown and Ransom, 2007). Astrocyte metabolism is critical in providing neurons with not only glucose, but also the glycolytic derivative lactate, as a means of metabolic support. Glucose is taken up by astrocytes through glucose transporters (GLUTs), and is subsequently converted into glycogen (Vannucci et al., 1997). Astrocytes represent a major, localized glycogen store in the brain, which can be utilized for metabolic support of neurons during periods of intense neuronal activity through the trafficking of glucose and lactate (Cataldo and Broadwell, 1986; Brown and Ransom, 2007).

Astrocyte metabolism and metabolic support of neurons remains a controversial topic in regards to the pathways and substrates involved. One view is that glucose is the sole substrate for energy production in neurons, while an opposing viewpoint includes astrocyte derived lactate as a substrate (Pellerin and Magistretti, 2003; Schurr, 2006). The currently held view of astrocyte metabolic support involves the astrocyte-neuron lactate shuttle hypothesis (ANLSH). This pathway was originally postulated by Pellerin and Magistretti (1994), in which astrocyte glycogen is broken down into lactate and shuttled to neurons to serve as aerobic fuel. Several lines of evidence now support the ANLSH as a critical pathway in the connection between astrocyte metabolism and neuronal activity (Brown and Ransom, 2007).
Recently, two studies provided direct functional imaging of astrocyte metabolism in response to neuronal activity. Kasischke et al. (2004) used two-photon imaging of hippocampal astrocyte to show that stimulation of Schaffer collaterals leads to an increase in astrocyte glycolysis measured by the intrinsic fluorescence of $\beta$-nicotinamide adenine dinucleotide (NADH). The authors concluded that this increase in glycolysis sustains neuronal oxidative metabolism through the ANLSH pathway. In a separate study in hippocampal slices, Rouach et al (2008) used a fluorescent glucose derivative to image the trafficking of glucose and glucose metabolites through the astrocyte syncytium via gap junction coupling. Changes in fluorescence in the astrocyte syncytium were coupled to neuronal activity, indicating that astrocytes are capable of shuttling glucose and glucose derivatives to site of neuronal metabolic demand. These studies provide strong evidence for the interaction between astrocytes and neurons in meeting the energy demands of the central nervous system.

In addition to being a major glycogen store in the brain and providing metabolic support for neuronal energy production, astrocytes are also involved in the synthesis and release of glutamine. Astrocytes express glutamine synthetase (GS), which produces glutamine from glutamate that has been taken up into astrocytes by glutamate transporter activity (Norenberg and Martinez-Hernandez, 1979). Glutamine produced by astrocytes is released into the extracellular space and taken up by neurons to be converted into the neurotransmitter glutamate (Hertz, 2006). Further, glutamine released by astrocytes is also taken up by inhibitory neurons and converted into GABA (Liang et al., 2006). It has been proposed that interference in
the glutamate-glutamine cycle in astrocytes rapidly leads to the depletion of synaptic GABA and may be involved in the generation of pathological processes (Ortinski et al., 2010). The involvement of astrocytes in providing glutamine to neurons constitutes another pathway by which astrocytes can modulate synaptic transmission in the central nervous system.

Synapse Formation and Dendritic Spine Interactions

Astrocytes are involved in the development and maturation of synapses in the central nervous system; however, for some time the mechanism was not known. The expression of soluble factors, extracellular matrix proteins and downstream signaling cascades in astrocytes has placed glial cells as key players in the development and maturation of synaptic contacts in many regions of the brain (Faissner et al., 2010). Astrocyte conditioned media in culture experiments can induce synapse formation in hippocampal neurons through a soluble factor released from astrocytes (Pfrieger and Barres, 1997; Nagler et al., 2001). For example, secretion of thrombospondins (specifically TSP1 and TSP2) from immature astrocytes increases synapse number in cultured neurons (Christopherson et al., 2005). Further, in vivo data revealed that TSP1/2 deficient mice display a reduced number of synapses, providing support for TPS1/2 having a role in synapse formation. It should be noted that while these synapses are structurally normal, they are postsynaptically silent, indicating that another mechanism is involved in triggering the trafficking of postsynaptic receptors to form functional synapses.
(Christopherson et al., 2005). A possible mechanism may be through release of tumor necrosis factor alpha (TNFα). TNFα released by astrocytes has been reported to induce trafficking of AMPA receptors to postsynaptic sites to increase excitatory synaptic strength (Beattie et al., 2002). Interestingly, while TSPs are able to induce synapse formation at glutamatergic synapses, they are not able to induce synaptogenesis of inhibitory connections (Hughes et al., 2010). A unrelated and yet unidentified secreted protein contained in astrocyte conditioned media appears to be involved. Therefore, astrocytes may be capable of selectively distinguishing and promoting synaptogenesis in varying neuronal cell types by release of distinct soluble factors.

Astrocytes are also involved in the maintenance of synaptic contacts and regulation of synaptic strength through ensheathment of synapses by fine processes. A single astrocytes is estimated to contact 300-600 dendrites and 100,000 synapses depending on the brain region examined (Bushong et al., 2002; Halassa et al., 2007b). The extent of astrocyte ensheathment varies between brain regions, with Bergmann glia nearly completely surrounding the parallel fiber-Purkinje cell synapse and only coverage of less than half of synaptic interface by astrocytes in the hippocampus (Grosche et al., 1999; Ventura and Harris, 1999). Astrocyte process interaction with dendritic spines is highly dynamic, with processes rapidly approaching and retracting (Haber et al., 2006). These dynamic changes suggest that astrocytes are capable of controlling spine maturation and stabilization through interaction with surface receptors.
Astrocytes can regulate synaptic strength through the interaction of astrocyte processes and dendritic spines involving signaling through ephrins and Eph receptors (Murai et al., 2003). Activation of EphA4 receptors on neuronal spines by ephrin-A3 on astrocytes processes can induce spine retraction in hippocampal dendrites. Further, this interaction can modulate LTP at CA3-CA1 synapses, not due to release of transmitters from astrocytes but due to changes in astrocyte glutamate transporter activity (Filosa et al., 2009). This represents a novel mechanism by which dynamic interaction of astrocyte processes and dendritic spines may regulate synaptic strength.

Astrocytes in Physiology and Pathology

As discussed above, astrocytes are actively involved in regulating synaptic activity and more widespread processes such as cerebral vascular responses. Recently, several studies concerning astrocytes regulating specific behavioral processes have been published. A dominant negative SNARE expressed in astrocytes was shown to block vesicular release of ATP/adenosine to affect the accumulation of the homeostatic sleep pressure (Halassa et al., 2009b). Further, this mouse model showed that astrocytes can affect slow cortical oscillations through reduced ambient adenosine tone and hypofunction of NMDA receptors (Fellin et al., 2009). Recently a novel role for astrocytes in the control of respiratory responses in the brainstem was published (Gourine et al., 2010). Astrocytes responded to changes in pH with intracellular Ca^{2+} increases that triggered the
release of ATP to activate chemoreceptor neurons and modify breathing. Taken together, the findings place astrocytes in position to regulate physiological processes in vivo.

Astrocytes have also been implicated in modulating physiological processes involved in addiction behaviors. Acute reward behavior of alcohol can be modulated by astrocytes. Astrocyte swelling triggers the release of taurine, an agonist of glycine receptors, and causes an increase in extracellular dopamine levels (Adermark et al., 2010). Endocannabinoids can trigger the Ca\(^{2+}\)-dependent release of glutamate from astrocytes to activate neuronal NMDA receptors, suggesting they may be involved in modulating endocannabinoid-related addiction (Navarrete and Araque, 2008). Finally, astrocytes are implicated in cocaine addiction through Ca\(^{2+}\)-dependent glutamate release from astrocytes onto neurons in the nucleus accumbens (Haydon et al., 2009).

Astrocytes are known to play a part in pathological states, primarily through the reactive gliosis. Astrocyte dysfunction leading to the onset or contributing to various pathological states has become an area of intense research. Investigations into pathological response have indicated a role for astrocytes in Alzheimer’s diseases, chronic pain, and epilepsy. In Alzheimer’s disease (AD), astrocytes surround plaques and are critical to the clearance of amyloid-\(\beta\) (Wegiel et al., 2001). Further, transgenic mice that express human A\(\beta\) precursor protein and mutant presenilin 1 display deregulated Ca\(^{2+}\) signaling, with increased frequency of Ca\(^{2+}\) transients (Kuchibhotla et al., 2009). Chronic pain has moved to the forefront of astroglial research in recent years, with evidence for the role of astrocytes in the
release of cytokines and chemokines in mediating neuropathic pain. While there is a growing role for activated astrocytes in the production of pathological pain, there is also evidence for protective roles as well (Milligan and Watkins, 2009).

The role of astrocytes in epilepsy has received much attention, with work in both acute hippocampal slices and in vivo implicating astrocytic involvement in the generation of epilepsy. However, the literature is not unified on a mechanism for how astrocytes may be regulating epilepsy. There is evidence for the involvement of gap junctions (Lee et al., 1995), loss of astrocytic domain structure (Oberheim et al., 2008), release of transmitters from astrocytes (Tian et al., 2005; Gomez-Gonzalo et al., 2010) and changes in the glutamate-glutamine cycle in reactive astrocytes (Ortinski et al., 2010). This is in addition to numerous functional changes displayed by astrocytes in response to epilepsy such as alterations in membrane channel expression; transporter and neurotransmitter receptors (Binder and Steinhauer, 2006).

Collectively, there is little doubt as to the involvement of astrocytes in pathological states and disease. Further investigation using in vivo techniques and genetic models will be crucial to determining what mechanisms in astrocytes are playing a role in the formation and maintenance of pathological disease.

1.10. Astrocyte Calcium Signaling

One of the most distinguishing features of astrocytes is that they are non-excitable cells. Astrocytes membrane currents are passive and they display no
ability to generate action potentials. Given these findings, the majority stance was that astrocytes were silent support elements in the brain. This viewpoint radically shifted when it was discovered that astrocytes both in culture and in situ express a wide variety of G protein coupled receptors (GPCRs) that could elicit downstream signaling cascades (McCarthy and de Vellis, 1978; van Calker et al., 1978; Porter and McCarthy, 1997). The most widely studied GPCR signaling cascade in astrocytes is the canonical phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP$_3$) pathway. Upon GPCR activation, PLC hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and IP$_3$, leading to IP$_3$ receptor (IP$_3$R) activation and Ca$^{2+}$ release from the endoplasmic reticulum (ER). This is based on an exhaustive amount of data from both cultured astroglia and in situ astrocytes and has been the subject of numerous reviews (Scemes, 2000; Parri and Crunelli, 2003; Volterra and Steinhauser, 2004; Fiacco and McCarthy, 2006; Scemes and Giaume, 2006).

In contrast to other glial cell types, there is surprisingly little data for alternate Ca$^{2+}$ signaling mechanisms in astrocytes in situ which has lead to a discrepancy between culture and in situ data. While cultured astrocytes express several voltage gated calcium channels (VGCC) (Latour et al., 2003; D'Ascenzo et al., 2004), there is less evidence for their functional role in situ. Astrocytic Ca$^{2+}$ elevations following stimulation of neuronal afferents are attributed to activation of astrocytic Gq-GPCRs and not to activation of VGCCs (Porter and McCarthy, 1995b, 1995a; Carmignoto et al., 1998; Nett et al., 2002; Beck et al., 2004; Straub and Nelson, 2007). However, while astrocytic VGCCs do not seem to play a role in the initiation of evoked
astrocytic Ca\textsuperscript{2+} increases, they may be important for initiating Ca\textsuperscript{2+} oscillations that occur independent of neuronal input, generally referred to as spontaneous or intrinsic astrocytic Ca\textsuperscript{2+} oscillations (Parri and Crunelli, 2001; Aguado et al., 2002). However, there is a possibility that specific subpopulations of astrocytes may express VGCCs. Recently evidence suggests that SVZ astrocyte-like cells express functional VGCCs that open in response to activation of GABA\textsubscript{A} receptors (Young et al., 2010). There is also indirect evidence for VGCCs on hippocampal astrocytes, where GABA\textsubscript{B} receptor activation (a Gi/o coupled receptor) was necessary for the release of ATP/adenosine from astrocytes (Serrano et al., 2006). While there is no electrophysiological data supporting expression of VGCCs in astrocytes in situ, the passive nature of the astrocyte membrane plus technical limitations on imaging in fine processes of astrocytes may occlude their detection.

Ryanodine receptors (RyRs) are another major class of ER Ca\textsuperscript{2+} release channels found in many cells types (Zalk et al., 2007). There is little convincing data that astrocytes in situ exhibit RyR-mediated increases in Ca\textsuperscript{2+} (Nett et al., 2002; Beck et al., 2004; Hertle and Yeckel, 2007). However, it remains possible that this alternate Ca\textsuperscript{2+} source could be important in the fine processes of astrocytes where it is difficult to study Ca\textsuperscript{2+} regulation.

Lastly, there is evidence for the expression of both Ca\textsuperscript{2+} permeable and impermeable AMPA receptors on astrocytes like those found in other glial subtypes such as Bergmann glia, oligodendrocytes and NG2 positive glia (Burnashev et al., 1992; Butt, 2006; Hamilton et al., 2010). However, this appears to occur only in specific astrocyte subpopulations such as those found in white matter tracks of optic
nerves and on GFAP positive astrocytes in the hippocampus that lack glutamate transporter currents (Zhou and Kimelberg, 2001; Hamilton et al., 2008). Therefore, based on the current state of the literature, the vast majority of Ca^{2+} signaling in astrocytes is mediated solely through Gq-GPCR activation and downstream activity of the PLC/IP_3 pathway.

Astrocytes express an exceptionally broad range of Gq-coupled GPCRs linked to the mobilization of intracellular Ca^{2+} through the PLC/IP_3 pathway (Agulhon et al., 2008). Receptors on astrocytes can be activated both by agonist application and by release from presynaptic terminals (Shelton and McCarthy, 1999) (Porter and McCarthy, 1995b, 1996; Pasti et al., 1997; Kang et al., 1998; Araque et al., 2002; Perea and Araque, 2005b; Navarrete and Araque, 2008). The ability of astrocytes to respond to neuronal activity with increases in intracellular Ca^{2+} provided evidence of a direct neuron to astrocyte signaling pathway, opening up speculation as to the role of this signaling modality in astrocytes and its functional consequences. The first report for a functional role came when Gq-GPCR agonists triggered intracellular Ca^{2+} release in astrocytes resulting in changes to ionotropic glutamate receptor (iGluR) activity on neurons (Parpura et al., 1994; Pasti et al., 1997). This astrocyte mediated change in neuronal activity occurs not only upon agonist stimulation of intracellular Ca^{2+} release, but can occur through spontaneous Ca^{2+} oscillations in astrocytes (Parri et al., 2001). These finding laid the ground work for one of largest paradigm shifts in neuroscience, with astrocytes releasing neurotransmitters (now referred to as gliotransmitters) to modulate and even drive neuronal activity in the brain.
1.11. The Tripartite Synapse

The tripartite synapse model is the theory that astrocytes, in response to neuronal activity, increase intracellular Ca\textsuperscript{2+} and release transmitters to affect synaptic transmission (Araque et al., 1999). The tripartite synapse is structurally composed of the presynaptic terminal, the postsynaptic bouton and an ensheathing astrocyte process (see Figure 1.1). This structural arrangement, in addition to the expression of GPCRs linked to Ca\textsuperscript{2+} mobilization, allows astrocytes to sense synaptic activity and respond with the release of what are termed gliotransmitters (Halassa et al., 2007a). The most commonly studied gliotransmitters are glutamate, ATP and D-serine; all of which are released from cultured astrocytes as well as astrocytes in situ (Hamilton and Attwell, 2010). This topic has been extensively reviewed, and therefore only a summary of the salient finding for gliotransmission will be presented below. For more comprehensive reviews see Agulhon et al. (2008) and Halassa et al. (2010).
1.12. Gliotransmitters and Modulation of Synaptic Activity

The currently defined gliotransmitters are glutamate, ATP (which is rapidly converted to adenosine in the extracellular space) and D-serine (Halassa et al., 2007a). The first of these gliotransmitters identified was glutamate. Gq-GPCR
stimulation of cultured astrocytes triggered release of glutamate in response to intracellular Ca\textsuperscript{2+} increases to activate neuronal NMDA receptors (Parpura et al., 1994). These results were corroborated by further experiments in neuron-astrocyte co-cultures and in acute hippocampal slices (Pasti et al., 1997; Araque et al., 1998b). This led to a series of high profile papers describing modulation of neuronal activity through the Ca\textsuperscript{2+}-dependent release of glutamate from astrocytes.

Glutamate release from astrocytes can activate presynaptic mGluR receptors (Fiacco and McCarthy, 2004; Andersson et al., 2007a; Perea and Araque, 2007) or pre- and postsynaptic NMDA receptors on excitatory neurons (Pasti et al., 2001; Angulo et al., 2004; Fellin et al., 2004; Jourdain et al., 2007; Navarrete and Araque, 2008).

Astrocyte Ca\textsuperscript{2+} -dependent release of glutamate is also implicated in modulating inhibitory neuron activity. Stimulation of astrocyte intracellular Ca\textsuperscript{2+} release leads to an increase in miniature inhibitory post synaptic currents (mIPSCs) in CA1 pyramidal neurons (Kang et al., 1998). Further, Ca\textsuperscript{2+}-dependent glutamate release from astrocytes can increase spontaneous IPSCs (sIPSCs) in hippocampal interneurons through activation of AMPA/KA receptors (Liu et al., 2004a).

Paradoxically, it was found upon blockade of action potential driven activity that intracellular Ca\textsuperscript{2+} increases in astrocytes lead to a reduction in the frequency of mIPSCs on interneurons and was not affected by AMPA/KA receptor blockers. These findings were later clarified when data indicated that Ca\textsuperscript{2+}-dependent release of glutamate from astrocytes also activated presynaptic group II/III mGluRs to inhibit presynaptic activity (Liu et al., 2004a). These findings implicate astrocyte Ca\textsuperscript{2+} -
dependent release of glutamate as a modulatory element of both excitatory and inhibitory synaptic transmission.

In the last decade, increasing evidence supporting the Ca$^{2+}$-dependent release of ATP from astrocytes has warranted its inclusion as a gliotransmitter. Upon release into the extracellular space ATP acts on purinergic receptors and is rapidly converted into adenosine by ectonucleotidases (Zimmermann, 2006). Activation of adenosine receptors, particularly A1 adenosine receptors (A$_1$R), is important in the regulation of presynaptic release at many synapses including CA3-CA1 and MF-CA3 synapses (Manita et al., 2004; Kukley et al., 2005). Cultured astrocytes are known to release ATP/adenosine in response to Gq-GPCR-mediated Ca$^{2+}$ increases (Pangrsic et al., 2007; Zhang et al., 2007).

Evidence in situ for astrocytic ATP release indicates that interfering with vesicular release of ATP through the expression of a dominant negative SNARE protein reduced the dynamic range of long term potentiation in dnSNARE mice (Pascual et al., 2005). Further, this blockade of ATP release prevented the formation of activity-dependent heterosynaptic depression at CA3-CA1 synapses. These finding were supported by a separate study, which also found that ATP released from astrocytes is involved in the formation of heterosynaptic depression (Serrano et al., 2006). However, while both these studies describe an effect on heterosynaptic depression, the pathways involved appear to differ. Pascual et al (2005) reported a SNARE-dependent release of ATP from astrocytes upon Schaffer collateral stimulation suggesting that astrocytes are responding to excitatory transmitter release from neurons. In contrast, Serrano et al (2006) provided data
that GABA released from interneurons triggered a GABA$_B$ receptor-dependent increase in astrocyte intracellular Ca$^{2+}$ to trigger the Ca$^{2+}$-dependent release of ATP. While the findings of the two papers do not directly contradict each other, they raise questions as to the neuronal pathways involved in astrocyte mediated regulation of heterosynaptic depression.

The third currently recognized, though less studied, gliotransmitter is D-serine. Astrocytes are proposed to be the sole source of D-serine released in the brain (Schell et al., 1997; Oliet and Mothet, 2006), though this stance has recently been challenged (Rosenberg et al., 2010). Several studies indicate that the enzyme responsible for synthesis of D-serine is highly localized to astrocytes in nearly all regions of the brain (Williams et al., 2006). Cultured astrocytes release D-serine in a Ca$^{2+}$ and vesicular-dependent manner upon stimulation of both ionotropic and metabotropic glutamate receptors (Schell et al., 1995; Mothet et al., 2005).

Currently the best evidence for astrocytic D-serine release in situ comes from studies in acute slices of the supraoptic nucleus (SON). Electrophysiological recordings of SON neurons indicate that D-serine levels in the synaptic cleft change as a function of astrocyte coverage of neurons (Panatier et al., 2006). These fluctuations in astrocyte derived D-serine levels were found to modulate NMDA receptor function, and affect synaptic processes such as LTP and LTD of SON neurons. The most recent study concerning D-serine release by astrocytes proposed an even more critical role in modulating synaptic transmission. Henneberger et al. (2010) found that by "clamping" intracellular Ca$^{2+}$ levels in astrocytes to block Ca$^{2+}$-dependent release of D-serine that formation of LTP at
CA3-CA1 synapses was completely blocked in the hippocampus. This effect was reversed by the addition of exogenous D-serine or glycine. It should be noted that this technique has not been widely used for the examination of Ca\(^{2+}\)-dependent release from astrocytes; therefore, further study will be required to place the findings of Henneberger et al. (2010) into a physiological context.

1.13. Release Mechanisms of Gliotransmitters

Studies using cultured astrocytes have provided evidence for a number of non-vesicular, gliotransmitter release mechanisms. Detectable gliotransmitter release through volume regulated anion channels (Takano et al., 2005; Abdullaev et al., 2006; Ramos-Mandujano et al., 2007), connexin hemichannels (Stout et al., 2002; Ye et al., 2003), and large conductance pores such as P2X\(_7\) receptors (Duan et al., 2003; Fellin et al., 2006a; Suadicani et al., 2006) has been reported. However, as astrocytes are known to alter their morphology and gene expression in culture, it remains unresolved which of these alternate mechanisms, if any, are physiologically relevant to gliotransmission in vivo (Hamilton and Attwell, 2010; Parpura and Zorec, 2010).

The most widely held mechanism for gliotransmitters release, particularly glutamate, is through IP\(_3\)R-meditated, Ca\(^{2+}\) and SNARE-dependent vesicular release (Halassa and Haydon, 2010). This view is based on findings that suggest, but are not conclusive, that astrocytes possess the mechanisms for vesicular release. Vesicular release by astrocytes is argued to be blocked in mice expressing a
dominant negative SNARE (dnSNARE) or by use of tetanus toxin to cleave synaptobrevin 2 (Pascual et al., 2005; Perea and Araque, 2007). However there is no evidence that vesicular release of glutamate is blocked in the dnSNARE, as expected in that model, only that exocytosis of ATP is affected. Tetanus toxin dialyzed into astrocytes via path pipette blocked the release of glutamate to affect synaptic strength, which the author interpreted as evidence of vesicular release (Perea and Araque, 2007). However, tetanus toxin may also affect the insertion and recycling of plasma membrane proteins involved in non-exocytotic, Ca\(^{2+}\)-independent release of gliotransmitters (Hamilton and Attwell, 2010). Further, glutamate transporter density at the plasma membrane is regulated by Ca\(^{2+}\)-dependent exocytosis and therefore could be affected by tetanus toxin and results in changes to synaptic activity (Stenovec et al., 2008).

There are conflicting reports on the expression of vesicular machinery between cultured and in situ astrocytes. Astrocytes in culture express several of the proteins associated with the cellular machinery for vesicular release such as SNAP-25 and syntaxin (Wilhelm et al., 2004; Zhang et al., 2004a; Montana et al., 2006). While Wilhelm et al. (2004) found abundant expression of release machinery in cultured astrocytes, expression in situ was lacking for several of the proteins found in culture. Gene array profiling of astrocytes from the cortex also found no evidence for the expression of several exocytotic machinery components (Cahoy et al., 2008). Further, there is no evidence for the expression of proteins such as Munc13 or Rab3, which are associated with exocytotic release in neurons, in astrocytes to date.
Finally, the presence of vesicles and the transporters necessary for their filling in astrocytes is not convincing. Astrocytes in situ display small, synaptic-like microvesicles, but their density is far less than found in neurons (Bezzi et al., 2004; Jourdain et al., 2007). Recent reports in acute hippocampal slices describe trafficking of vesicles in astrocytes, but there is little evidence that these are vesicles involved in regulated Ca\(^{2+}\)-dependent exocytosis of gliotransmitters (Potokar et al., 2009). Further, the expression of vesicular glutamate transporters (VGLUTs) in astrocytes is debatable. There is evidence both for and against the expression of VGLUT1 and 2 in situ (Bezzi et al., 2004; Zhang et al., 2004b; Potokar et al., 2009), with expression of VGLUT3 confined to a subpopulation of white matter astrocytes (Fremeau et al., 2002). It should be noted that there is abundant evidence that VGLUT1 and 2 are solely expressed by asymmetric terminals of excitatory neurons (Gras et al., 2002; Herzog et al., 2006). Based on the available evidence, it seems unlikely that astrocytes possess the necessary cellular machinery for Ca\(^{2+}\)-dependent exocytosis of vesicles on the time scale required for gliotransmission as it is currently defined (Fiacco et al., 2009).

The theory of vesicular release from astrocytes, in spite of the evidence against it, remains an integral part of astrocyte modulation of neuronal activity, regardless of the gliotransmitters studied or the brain region examined. No matter the release mechanism involved, the primary unifying feature to nearly all studies describing neuromodulation by astrocytes in situ and in vivo is IP\(_3\)R-mediated intracellular Ca\(^{2+}\) increases that are deemed necessary and sufficient to trigger the release of gliotransmitters (Parpura and Haydon, 2000). Further, GPCR activation
of the PLC/IP₃ pathway and Ca²⁺ release through IP₃Rs is generally considered to be the responsible for intracellular Ca²⁺ elevations triggering the release of gliotransmitters.

1.14. The Stirrings of Controversy

One persistent problem exists in the study of gliotransmission: How does one selectively stimulate intracellular Ca²⁺ increases in astrocytes without causing direct stimulation of neurons? Astrocytes express an extensive compliment of GPCRs, the vast majority of which are found also on neurons (Agulhon et al., 2008). Therefore, application of Gq-GPCRs agonists to elicit astrocyte Ca²⁺ increases runs a high probability of causing direct changes to neuronal activity by activating the same GPCRs on neurons (Pasti et al., 1997; Lee et al., 2007; Shigetomi et al., 2008). An example is the use of DHPG, a group 1 mGluR agonist that was used to show astrocyte derived effects on neurons (Angulo et al., 2004; Fellin et al., 2004). Group 1 mGluRs are also expressed by neurons, where they can cause increases in Ca²⁺ and activation of NMDA receptors, all things that have been attributed to gliotransmission (Mannaioni et al., 2001; Choe et al., 2006). While this method of stimulation is still used for the study of gliotransmission, unless extensive controls are conducted it remains difficult to distinguish between direct neuronal effects and astrocyte-derived effects on synaptic activity.

Alternative methods for selectively activating astrocytes have been used by the field in recent years, each with their own technical caveats. These include
mechanical stimulation of astrocytes, where a pipette is used to contact the surface of the astrocyte to elicit intracellular Ca\(^{2+}\) increases and gliotransmitters release (Kozlov et al., 2006; Hamilton et al., 2010). However, mechanical stimulation can potentially be damaging to the cell membrane, and is certainly not physiological.

Intense depolarization of astrocytes through either patch clamp electrodes or application of high potassium solutions has been used to demonstrate intracellular Ca\(^{2+}\) increases in astrocytes and release of glutamate (Jourdain et al., 2007; Yaguchi and Nishizaki, 2010). However, depolarization of astrocytes can interfere with the electrogenic drive of glutamate transporters, resulting in reduced clearance from the synaptic cleft and transmitter spillover to activate extrasynaptic receptors on neurons (Danbolt, 2001). Further, high potassium solutions will result in depolarization of synaptic terminals to drive exocytosis from neurons.

The most commonly used methods of selectively increasing intracellular Ca\(^{2+}\) in astrocytes is the uncaging of either Ca\(^{2+}\) or IP\(_3\) in astrocytes. Astrocytes can be patch loaded with a salt caged form of Ca\(^{2+}\) or IP\(_3\), which can be activated in response to UV laser light. Numerous reports have used these methods to show that selective increases in astrocyte intracellular Ca\(^{2+}\) result in the release of gliotransmitters (Fellin et al., 2004; Fiacco and McCarthy, 2004; Perea and Araque, 2007; Gordon et al., 2009). While these methods do allow researchers to selectively increase Ca\(^{2+}\) in astrocytes, the Ca\(^{2+}\) increases that result from these techniques are spatially and temporally distinct from endogenous responses.
Uncaging of Ca\textsuperscript{2+} increases intracellular Ca\textsuperscript{2+} beyond what is seen normally by Gq-GPCR activation, and it does not engage a number of second messenger systems activated by GPCRs. For example, this method bypasses the activation of PLC, which produces not only IP\textsubscript{3} but diacylglycerol (DAG). Downstream effects of DAG include the activation of protein kinase C (PKC), which can modulate vesicular release (Lin et al., 2009; Kelm et al., 2010) and can modulate astrocyte intracellular Ca\textsuperscript{2+} increases (Parri and Crunelli, 2003). IP\textsubscript{3} uncaging activates the endogenous ER receptor, but produces supra-physiological levels of IP\textsubscript{3} that modify the response of the IP\textsubscript{3} receptor itself. In saturating levels of IP\textsubscript{3}, the IP\textsubscript{3}R becomes less sensitive to inhibition by Ca\textsuperscript{2+}, thereby increasing the open probability of the channel resulting in a prolonged Ca\textsuperscript{2+} increase (Foskett et al., 2007). While these methods appear to be the best solution, uncaging of Ca\textsuperscript{2+} or IP\textsubscript{3} results in a nonphysiological level of Ca\textsuperscript{2+} that may result in a nonphysiological release of gliotransmitters.

The methods for the blockade of intracellular Ca\textsuperscript{2+} increases in astrocytes are less problematic. Inhibition of IP\textsubscript{3}R through selective antagonists such as herparin (Ehrlich et al., 1994) or xestospongins (Gafni et al., 1997) is possible, but for most studies into gliotransmission this is not ideal as these blockers will also inhibit neuronal IP\textsubscript{3}Rs and make data interpretation problematic. The most commonly used method of blocking intracellular Ca\textsuperscript{2+} increases in astrocytes is through the use of Ca\textsuperscript{2+} chelators such as BAPTA. The overwhelming majority of studies into the Ca\textsuperscript{2+}-dependent release of gliotransmitters involve the use of patch loaded or bulk loaded BAPTA to block intracellular Ca\textsuperscript{2+} increases in astrocytes. While BAPTA chelation is an effective method, it may not block Ca\textsuperscript{2+} increases in fine processes of astrocytes.
nor does it specifically address IP₃R-mediated Ca²⁺ increases. Further, it is likely that bulk loaded BAPTA affects neuronal as well as astrocytic Ca²⁺ fluxes.

Recently, two genetic models were developed to address these issues. The first is the MrgA1 receptor mouse model published by Fiacco et al (2007). This mouse model uses an inducible promoter to drive astrocytic expression of the orphan receptor MrgA1, normally found in a specific subset of sensory neurons in the spinal cord and whose endogenous agonist is unknown (Han et al., 2002). MrgA1 is a Gq-linked GPCR that responds to synthetic RF amide neuropeptides with increases in intracellular Ca²⁺ from internal IP₃R-mediated stores (Dong et al., 2001). This allows selective activation of astrocytic intracellular Ca²⁺ increases through bath application of a synthetic agonist without any direct effects on neuronal Gq-GPCRs.

In a seminal paper by Fiacco et al (2007), selective activation of astrocyte intracellular Ca²⁺ increases was found to be unable to produce changes in CA1 pyramidal neuron excitatory synaptic activity. Further, use of endogenous ligands to endothelin receptors, which are highly expressed by astrocytes in the brain (Schinelli et al., 2001; Ostrow and Sachs, 2005), was unable to affect neuronal NMDA mEPSCs. However, uncaging of IP₃ in MrgA1 astrocytes caused the release of glutamate from astrocytes to increase AMPA sEPSC frequency of CA1 pyramidal neurons (Fiacco et al., 2007). This was a critical finding of the study as it showed that MrgA1+ astrocytes could be driven to release glutamate using non-physiological methods but that selective activation of both exogenous and endogenous Gq-GPCRs do not trigger release. These findings called into question the role of Gq-GPCRs and IP₃ receptors in the theory of gliotransmission.
The second model is the IP$_3$R2 knockout (KO) mouse model. These mice were originally developed to study the role of IP$_3$Rs in Ca$^{2+}$ signaling of atrial myocytes (Li et al., 2005). In astrocytes, immunohistochemical and gene profiling data suggested that IP$_3$R type 2 (IP$_3$R2) was the primary IP$_3$R expressed by astrocytes (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007; Cahoy et al., 2008). Evidence for IP$_3$R2 in astrocytes and its role in generating intracellular Ca$^{2+}$ transients had only been shown in cultured astrocytes, and did not rule out functional expression of other IP$_3$R isoforms (Sheppard et al., 1997; Weerth et al., 2007).

In Petravicz et al (2008), the first functional data in situ that Gq-GPCR activation of astrocytic intracellular Ca$^{2+}$ increases is solely mediated by IP$_3$R2 was presented. Calcium imaging studies in mice lacking IP$_3$R2 expression revealed that bath application of several Gq-GPCR agonists was completely incapable of eliciting a Ca$^{2+}$ response in astrocytes. Further, lack of IP$_3$ receptor mediated Ca$^{2+}$ increases in astrocytes did not affect basal excitatory synaptic activity, contrary to predictions made from computer models using experimental evidence of gliotransmission (Nadkarni and Jung, 2007). The findings in this paper combined with the findings of Fiacco et al (2007), present a startling viewpoint that Gq-GPCR-mediated, IP$_3$R-dependent Ca$^{2+}$ increases in astrocytes may not be involved in the release of gliotransmitters to modulate neuronal activity.

Recently, an additional paper from the McCarthy lab utilized both the MrgA1 and IP$_3$R2 KO mouse models to investigate the role of Gq-GPCR-mediated, IP$_3$R-dependent Ca$^{2+}$ increase in astrocytes on the formation of hippocampal short- and
long-term potentiation (Agulhon et al., 2010). No effect of either selectively activating (MrgA1) or selectively blocking (IP$_3$R2 KO) was found on the formation of short- or long-term potentiation of CA3-CA1 synapses. This directly contradicts reports that release of gliotransmitters from astrocytes modulates synaptic potentiation and the formation of LTP in the hippocampus (Pascual et al., 2005; Perea and Araque, 2007; Henneberger et al., 2010). This series of papers from the McCarthy laboratory illustrate that there needs to be a critical reevaluation of the theory of gliotransmission. A key area that needs reevaluation is the role of IP$_3$Rs as a physiological Ca$^{2+}$ source for Ca$^{2+}$-dependent release from astrocytes. In the next section, we will discuss IP$_3$R in general and more specifically the findings concerning IP$_3$R2 relevant to astrocytes.

1.15. Inositol Trisphosphate Receptors: Structure and Function

Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are a major class of endoplasmic reticulum (ER) proteins responsible for the release of Ca$^{2+}$ from internal ER-based stores in the majority of mammalian cell types. There are three genes (itpr1, itpr2 and itpr3) that encode three IP$_3$Rs (Ross et al., 1992). Each of the IP$_3$R genes displays multiple mRNA splice variants, with itpr1 being the most heavily spliced and iptr2 only having two known splice variants (Iwai et al., 2005; Foskett et al., 2007). The IP$_3$R protein is large, having a molecular mass of ~300kDa, and forms into a homo- or heterotetramer (Regan et al., 2005). Given the diversity of splice variants and formation of heterotetramers, this can potentially result in a wide
array of channel compositions with differing biophysical, spatial and temporal properties.

The IP₃R protein itself has four domains: an N-terminal IP₃ binding region, a coupling domain, a transmembrane domain and a C-terminal tail. The N-terminal IP₃ binding region is further broken down into a core and suppressor domain, both of which are required for IP₃ to induce conformational changes needed for channel gating (Mignery and Sudhof, 1990; Yoshikawa et al., 1996). Deletions in this region can either prevent channel gating, or interfere with binding of IP₃ to the receptor. The coupling domain is around 1700 amino acids and contains a large number of sites for phosphorylation, regulatory protein interactions, and modulation by ATP and Ca²⁺ (Patterson et al., 2004). The transmembrane domain contains the channel pore and activation gate that allows the flow of Ca²⁺ down its electrochemical gradient from the ER lumen to the cytoplasmic compartment (Taylor et al., 2004). Lastly, the C-terminal tail is functional site for modulation by several interacting proteins, primarily resulting in enhancement of IP₃R binding affinity for IP₃ (Patterson et al., 2004). The C-terminal also interacts with the N-terminal ligand binding region as part of the activation of the IP₃R (Nakade et al., 1991).

While the receptor is named after its IP₃ agonist, the channels itself requires both IP₃ and Ca²⁺ as co-agonists to fully activate (see review by Foskett et al., 2007). This dependence gives the receptor a dynamic range of activation, since channel gating depends on both the relative concentrations of IP₃ and Ca²⁺ available at a given time point and the receptor’s sensitivity to these co-agonists. This can be regulated by Ca²⁺ itself, as well as the vast array of interacting proteins and
phosphorylation states which affect the channel mean opening rate (Patterson et al., 2004). Due to the extensive regulatory domains it possesses, IP₃Rs function as signal integrators that possess the ability be modified by signaling networks to produce spatially and temporally distinct Ca²⁺ events. In addition, IP₃Rs can also act as scaffolding proteins, binding to GPCR complexes at the plasma membrane to tether and coordinate the signaling complex to ER calcium release sites (Fagni et al., 2002; Weerth et al., 2007).

1.16. Inositol Trisphosphate Receptors: Expression in Neurons and Astrocytes

IP₃Rs are expressed throughout all regions of the brain and spinal cord. Individual cell types in the CNS may express multiple IP₃Rs, providing functional redundancy or dynamic Ca²⁺ signals in cell types expressing more than one IP₃R subtype. Immunocytochemical and mRNA in situ hybridization studies have reported that the majority of neurons in the brain express primarily IP₃R1 and to varying degrees IP₃R3 (Fujino et al., 1995; Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007). Functional evidence that neurons in the brain express IP₃R1 comes from the generation of an IP₃R1 knockout mouse model (Matsumoto et al., 1996). This mouse is near embryonic lethal, with very few IP₃R1 KO pups being born. Pups that are born typically die by p14-p21 and suffer from seizures and ataxia. Additionally, neuronal cultures made from IP₃R1 KO pups show that these
neurons have severely impaired Ca\textsuperscript{2+} handling ability that contributes to the behavioral phenotype (Hayashi et al., 1999; Matsumoto and Kato, 2001).

In contrast to neurons, glial cells express primarily IP\textsubscript{3}R2 in the brain. Astrocytes solely express IP\textsubscript{3}R2 with no significant detection of IP\textsubscript{3}R1 or IP\textsubscript{3}R3 expression in immunocytochemistry studies from brain sections (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007). This is supported by gene array data of acutely isolated astrocytes from the cortex of mice (Cahoy et al., 2008). Further support for expression of IP\textsubscript{3}R2 and its primary role in generating astrocyte Ca\textsuperscript{2+} signals comes from cultured astrocytes, where clusters of IP\textsubscript{3}R2 correspond to Ca\textsuperscript{2+} release sites (Sheppard et al., 1997) and that IP\textsubscript{3}R2 associates with other components of the Gq-GPCR-PLC pathway in membrane lipid rafts through its ability to bind to Homer (Weerth et al., 2007).

1.17. Functional Aspects of IP\textsubscript{3}R2 and Their Role in Astrocyte Ca\textsuperscript{2+} Signaling

The isoforms of the IP\textsubscript{3}R display some similarities and differences in their functional activation and structural properties. IP\textsubscript{3}R2 shares a 70% homology with IP\textsubscript{3}R1, and a higher degree of homology with IP\textsubscript{3}R3 (Bezprozvanny, 2005). Among the IP\textsubscript{3}Rs, IP\textsubscript{3}R2 is the least understood isoform, relative to what is known for IP\textsubscript{3}R1 and IP\textsubscript{3}R3. However, there are several known properties of IP\textsubscript{3}R2 that may be important in their functional role as the primary Ca\textsuperscript{2+} release mechanism in astrocytes.
Functional evidence indicates that the IP₃R2 is involved in the generation of long lasting, regular Ca²⁺ increases, as determined by studies using a DT40 chick B cell line that has multiple IP₃R deletions (Miyakawa et al., 1999). This is attributed to the IP₃R2 not exhibiting appreciable inhibition in the presence of high Ca²⁺ concentrations, possessing a mid-range affinity (in relation to other IP₃Rs) for Ca²⁺, and the highest affinity for IP₃ among IP₃Rs (Ramos-Franco et al., 1998b; Ramos-Franco et al., 1998a; Tu et al., 2005). This combination of high IP₃ affinity, mid-range Ca²⁺ affinity and lack of significant inhibition in high Ca²⁺ concentrations would result in increased open probability and prolonged channel openings that could explain long time scale (tens of seconds) of astrocyte Ca²⁺ increases.

Activity-dependent and independent clustering of IP₃R has been observed for IP₃R2 (Iwai et al., 2005). Both activity-dependent and independent clustering is a function of IP₃ or Ca²⁺ binding to IP₃Rs, though there is disagreement over IP₃ or Ca²⁺ being the key binding event. Activity independent clustering of IP₃R2 may be attributable to the receptors high IP₃ affinity, thereby causing clustering at basal IP₃ levels. This promotes stable IP₃R2 cluster formation, which underlies elemental Ca²⁺ events such as Ca²⁺ puffs (Smith et al., 2009). Finally, in addition to IP₃R2 there are two identified splice variants, designated TIPR and IP₃R2 SIm²⁻. TIPR is found only in heart and skeletal muscle (Futatsugi et al., 1998), while the IP₃R2 SIm²⁻ splice variant shows a more ubiquitous expression pattern (Iwai et al., 2005). IP₃R2 SIm²⁻ produces a IP₃R2 subunit that is incapable of binding IP₃, displays no Ca²⁺ channel activity when expressed as a homotetramer, and interferes with activity-dependent clustering of IP₃R2. Further, this splice variant significantly
reduces ATP-induced \( \text{Ca}^{2+} \) entry without affecting \( \text{Ca}^{2+} \) release in cells; however, the entry mechanism involved was not investigated. This provides a possible functional role for IP\(_3\)R2 SIm\(^2_-\) in regulating store operated \( \text{Ca}^{2+} \) entry, which can affect the temporal dynamics of late phase \( \text{Ca}^{2+} \) signals (Golovina and Blaustein, 2000; Iwai et al., 2005).

Taken together, these biophysical properties fit well with the data concerning IP\(_3\)R-mediated \( \text{Ca}^{2+} \) increases in astrocytes (Fiacco and McCarthy, 2006). Astrocytic IP\(_3\)R2-mediated \( \text{Ca}^{2+} \) increases are typically longer lasting (on the time scale of seconds) and more resistant to desensitization than IP\(_3\)R1 mediated neuronal responses, which reflects the different biophysical properties of IP\(_3\)R2 versus IP\(_3\)R1 (Foskett et al., 2007). The high affinity of IP\(_3\)R2 may be involved in the frequency and rhythmic nature of IP\(_3\)R-mediated spontaneous \( \text{Ca}^{2+} \) events found in several populations of astrocytes (Parri and Crunelli, 2001; Nett et al., 2002; Wang et al., 2006).

Calcium microdomains exist in astrocytes, where discrete sites of \( \text{Ca}^{2+} \) release can be detected in the absence of a global response (Fiacco and McCarthy, 2006). This was demonstrated to occur both in cultured and \textit{in situ} astrocytes in response to Gq-GPCR agonist application and low-frequency electrical stimulation (Sharp et al., 1999; Matyash et al., 2001; Fiacco et al., 2007). Calcium microdomains in astrocytes may represent activation of stable, local clusters of IP\(_3\)R2 that are not of sufficient amplitude to propagate the \( \text{Ca}^{2+} \) signal past the diffusion barrier to reach the next cluster of IP\(_3\)Rs. This has been demonstrated to occur in neuronal dendrites, where localized \( \text{Ca}^{2+} \) signal can fail to propagate due to
the distance to the next IP₃R cluster (Fitzpatrick et al., 2009). Lastly, it is interesting to note that the IP₃R2 SIm²⁻ splice variant of IP₃R2 comprises ~20% of the total IP₃R2 mRNA in the brain (Iwai et al., 2005). While the localization for the mRNA of this splice variant in the brain has not been addressed, it is not unreasonable to speculate that it is functionally expressed in astrocytes and may play role in regulation of IP₃R-mediated Ca²⁺ increases.

1.18. Goals of Dissertation

In summary, a primary functional role assigned to IP₃R-mediated Ca²⁺ signaling in astrocytes is to trigger the release of gliotransmitters in response to neuronal activity. The theory of gliotransmission, where modulation of synaptic activity (both excitatory and inhibitory) in a Ca²⁺-dependent manner from astrocytes is currently wholly reliant on the Gq-GPCR-PLC-IP₃ pathway and functional activation of IP₃Rs in astrocytes. However, despite numerous lines of evidence that astrocytes modulate synaptic activity by releasing gliotransmitters in an IP₃R-mediated, Ca²⁺-dependent manner, the pathway has never been selectively blocked at the level of the IP₃R. The field has relied on the non-specific block of Ca²⁺ increases by Ca²⁺ chelators such as BAPTA, which blocks all Ca²⁺ sources in astrocytes. Therefore, a fundamental knowledge gap exists concerning the specific role of astrocytic IP₃R-mediated responses in gliotransmission and physiology.

To determine if astrocyte gliotransmitters release is dependent upon Ca²⁺ signaling through the Gq-GPCR-PLC-IP₃R pathway, several novel genetic knockout
mouse models were used. First, an IP₃R2 full knockout (IP₃R2 KO) mouse model was used to determine if genetic ablation of IP₃R2 blocks astrocytic Ca²⁺ increases in response to Gq-GPCR stimulation and its affect on basal CA1 pyramidal neuron activity in the hippocampus. Second, an IP₃R2 conditional knockout (IP₃R2 cKO) mouse model was generated to restrict the deletion of IP₃R2 to the CNS. The IP₃R2 cKO was used to expand on the studies in the IP₃R2 KO model and for an analysis of the result of blocking IP₃R2-mediated release in astrocytes on physiological behaviors. The findings of these studies provide valuable insight into the role of astrocytic IP₃R-mediated Ca²⁺ increases in gliotransmission and the regulation of physiological processes governing behavior.
Chapter II: Loss of IP$_3$ receptor-dependent Ca$^{2+}$ increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity.

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2.1. INTRODUCTION

Astrocytes have recently been reported to have a functional role in neuronal excitability (Araque et al., 1998a; Haydon and Carmignoto, 2006), heterosynaptic depression (Pascual et al., 2005; Serrano et al., 2006; Andersson et al., 2007b), cerebrovascular dynamics (Zonta et al., 2003; Straub and Nelson, 2007) and pathological states such as epilepsy (Kang et al., 2005; Tian et al., 2005; Fellin et al., 2006b). One unifying feature of these findings is that astrocyte modulation of these processes occurs via a Ca$^{2+}$-dependent release of “gliotransmitters” including
ATP (which is converted to adenosine by ectonucleotidases) and glutamate (Montana et al., 2006). Astrocytes primarily utilize spatially and temporally encoded increases in Ca\(^{2+}\) as an intracellular signaling mechanism (Cornell-Bell et al., 1990; Jensen and Chiu, 1990; Scemes and Giaume, 2006). Astrocytes display Ca\(^{2+}\) increases both spontaneously (Parri et al., 2001; Nett et al., 2002; Hirase et al., 2004) and in response to neuronal stimulation (Porter and McCarthy, 1996; Aguado et al., 2002; Perea and Araque, 2005a) and have been reported to modulate synaptic transmission through activation of both metabotropic (mGluR) and ionotropic glutamate receptors (iGluRs), and adenosine receptors (Haydon and Carmignoto, 2006).

Calcium increases in astrocytes are predominantly elicited by G\(_{q}\)-linked GPCR activation, driving the production of IP\(_3\) and the activation of IP\(_3\)Rs coupled to endoplasmic reticulum (ER) Ca\(^{2+}\) stores. IP\(_3\)Rs are a family of genes expressing three isoforms (Types 1-3) of an ER Ca\(^{2+}\) release channel that are found in nearly every cell type (Foskett et al., 2007). Immunohistochemical studies aimed at identifying the expression profile of IP\(_3\)Rs in the brain suggest that hippocampal astrocytes express primarily IP\(_3\)R2. Evidence for IP\(_3\)R2 expression in neurons is inconclusive (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007). These data point to IP\(_3\)R2 as a potential key mediator of astrocyte intracellular Ca\(^{2+}\) release and Ca\(^{2+}\)-dependent signaling cascades, but functional evidence for IP\(_3\)R2 in astrocytes is limited and has not been demonstrated \textit{in situ} (Sheppard et al., 1997; Weerth et al., 2007).
We used an IP$_3$R2 KO mouse model to determine if IP$_3$R2 has a functional role in Ca$^{2+}$ increases of hippocampal astrocytes and neurons of the CA1 region. We present the novel finding that genetic deletion of IP$_3$R2 results in complete loss of spontaneous and agonist evoked IP$_3$R-dependent Ca$^{2+}$ increases in astrocytes but leaves intact agonist evoked IP$_3$R-dependent Ca$^{2+}$ increases in CA1 pyramidal neurons. These data indicate that IP$_3$R2 is the primary functional IP$_3$R in astrocytes, and that IP$_3$R2 does not play a demonstrated role in CA1 pyramidal neurons. We performed electrophysiological recordings of CA1 pyramidal neuron sEPSCs to determine the effect of eliminating astrocytic Ca$^{2+}$ responses on basal neuronal excitatory synaptic activity. No significant changes were found in any of the AMPA-R and NMDAR sEPSC parameters of mice lacking astrocytic Ca$^{2+}$ responses compared to littermate controls.

Our results indicate that Gq-GPCR-mediated, IP$_3$R-dependent Ca$^{2+}$ responses in astrocytes are not modulating basal neuronal excitatory synaptic activity, contrary to the current state of the literature. We also found no differences in activation of NMDARs by ambient glutamate, providing additional evidence that ambient glutamate of glial origin is not released in a Ca$^{2+}$-dependent manner (Jabaudon et al., 1999; Cavelier and Attwell, 2005). This study provides the first functional evidence that IP$_3$R2 is the only IP$_3$R isoform expressed by astrocytes; that IP$_3$R2 is not required for neuronal Gq-GPCR-mediated Ca$^{2+}$ elevations, and that removal of astrocyte Ca$^{2+}$ increases has no effect on basal neuronal excitatory activity.
2.2. MATERIALS AND METHODS

Generation of IP₃R2 KO mice

IP₃R2 KO mice were generated as previously described (Li et al., 2005). Briefly, a 539-bp fragment of exon 3 of IP₃R2 (116 bp) was inserted into a targeting vector between two loxP sites. The targeting vector was injected into R1 embryonic stem (ES) cells, screened for homologous recombination and injected in C57Bl/6 blastocystes to generate male chimeras. Male chimeras were bred to Black Swiss females to generate mice heterozygous for the floxed allele (IP₃R₂²⁺/loth). IP₃R₂²⁺/loth mice were crossed to Pro-Cre mice to obtain mice double heterozygous for the floxed IP₃R2 and Pro-cre alleles. Pro-Cre, IP₃R₂²⁺/loth were crossed to generate germline heterozygous null mutant offspring (IP₃R₂²⁺⁺), which were interbred to generate homozygous full mutant mice (IP₃R₂²⁺⁺) and littermate controls (IP₃R₂²⁺⁺ and IP₃R₂²⁺⁺). Mice were genotyped by PCR analysis using genomic DNA with IP₃R2 WT and mutant allele specific primers as given in Li et al (2005).

Hippocampal slice preparation

All procedures followed the guidelines of the IACUC of UNC-Chapel Hill. Littermate control and IP₃R2 knockout mice 10 to 16 days old (P10-16) were anesthetized by isoflurane inhalation. The brains were rapidly removed after decapitation and submerged into 4°C slicing buffer containing the following in mM: 125 NaCl, 10 glucose, 1.25 NaH₂PO₄, 26 NaCHO₃, 2.5 KCl, 3.8 MgCl₂ and 0.1 kynurenic acid and bubbled with 95% O₂ and 5% CO₂. Brains were cut sagittally at
a thickness of 300 μm on a Leica (Bannockburn, IL) vibratome. During sectioning brains were kept submerged in 4°C oxygenated slicing buffer. Hippocampi were dissected out of each brain slice and incubated 45 min in artificial cerebral spinal fluid (ACSF) warmed to 35-37°C and bubbled continuously with 95% O₂ and 5% CO₂. The ACSF contained the following in mM: 125 NaCl, 10 glucose, 1.25 NaH₂PO₄, 26 NaCHO₃, 2.5 KCl, 2.5 CaCl₂, and 1.3 MgCl₂.

Calcium imaging

Astrocytes were bulk loaded with either the Ca²⁺ indicator Calcium Green-1 AM or Fluo-4 AM as previously described (Nett et al., 2002). Slices were incubated for 45 min at 35-37°C in oxygenated ACSF that included either 16 μM Fluo-4 AM or 11μM Calcium Green 1-AM ester dye and 0.07% pluronic acid (final DMSO concentration, 0.4%). For measuring neuronal Ca²⁺ increases, CA1 pyramidal neurons were patch-clamped with 200 μM Alexa 568 and 400 μM Fluo-4 calcium indicator dye made up in neuronal internal solution (see below). The pipette was then removed and the neuron was allowed to recover for 10 min. Regions of interested were placed over the cell bodies of astrocytes and over the cell body and primary dendrite of CA1 pyramidal neurons. Increases in average fluorescence in regions of interest indicate increase in Ca²⁺ concentration. Fold increase over baseline was calculated for each trace and reported as ∆F/F₀.
Neuronal patch clamp recordings

For AMPA-R sEPSC recordings, CA1 pyramidal neurons were patch clamped using pipettes (4.0-6.0 MΩ resistance) and a gap free recording was performed for 10 min in ACSF as previously described (Fiacco and McCarthy, 2004). For NMDA sEPSCs, neurons were voltage-clamped at -70 mV and superfused with ACSF containing 5 µM Mg²⁺ and 10 µM CNQX to block AMPA responses. For the ambient glutamate recordings, neurons were patch clamped at a holding potential of +40 mV in normal ACSF with an internal solution containing the following in mM: 100 cesium methanesulfonate (CH₃CsO₃S), 10 TEACl, 4 NaCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 5 phosphocreatine, 2 ATP, 0.3 GTP, the pH was adjusted to 7.3 with CsOH. Ambient glutamate current was recorded in ACSF containing 1 µM TTX and 100 µM picrotoxin.

Data Collection and Analysis

Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Traces were analyzed for sEPSCs in Clampfit 10.2 software (Axon Instruments) using a template constructed from 4-6 sEPSCs intrinsic to each recording. The event statistics were taken for each individual event and then averaged. The averaged event statistics from each cell were then averaged together and reported as mean ± SEM. For tonic NMDA currents from ambient glutamate recordings, membrane currents were normalized to the amplitude of the currents blocked by a saturating concentration of D-AP5. Statistical differences
between two samples were evaluated using Student’s t-test performed using Prism 4 software (Graphpad, San Diego, CA).

Histology

Littermate control and IP$_3$R2 KO brains were fixed with formalin and embedded sagittally in paraffin. Brains were sectioned at 6 $\mu$m thickness with a Leica (Nussloch, Germany) microtome from p30 littermate controls and IP$_3$R2 KOs. Sections were stained with hematoxylin and eosin and imaged using a Zeiss (Oberkochen, Germany) Axioscope light microscope.

Reagents

D-AP5, tetrodotoxin (TTX), CNQX, DHPG, histamine, carbachol, thapsigargin, DL-TBOA, and picrotoxin were obtained from Tocris Biosciences (UK). Calcium Green-1 AM, Fluo-4, Fluo-4 AM and Alexa 568 were obtained from Invitrogen (Carlsbad, CA).
2.3. RESULTS

**Histological analysis of IP₃R2 KO mouse brains**

It has been previously reported that mice homozygous for the IP₃R2 KO allele are viable and fertile and that the mice display no overt behavioral abnormalities (Li et al., 2005). We performed histological staining of paraffin embedded sections from adult IP₃R2 KO mice to determine if there were any obvious abnormalities in brain cytoarchitecture indicative of improper proliferation or neurite outgrowth during development. Hematoxylin and eosin staining of P30 littermate control (n = 3) and IP₃R2 KO (n = 3) brains revealed that lack of IP₃R2 does not significantly affect brain cytoarchitecture. The hippocampus, cortex and cerebellum of IP₃R2 KO mice were examined and did not show any obvious structural abnormalities (Figure 2.1). These findings suggest that IP₃R2-mediated release of Ca²⁺ is not critical to the overall development of the brain or that alternate IP₃Rs may be expressed in glial cells during development.

**IP₃R2 KO hippocampal astrocytes lack spontaneous and Gq-linked GPCR Ca²⁺ increases**

Astrocytes are known to respond to a wide variety of Gq-GPCR agonists with Ca²⁺ elevations (Verkhratsky and Kettenmann, 1996). To address the functional role of IP₃R2 in hippocampal astrocytes, we performed Ca²⁺ imaging experiments on bulk loaded slices from littermate control and IP₃R2 KO mice. Bath application of a Gq-linked GPCR agonist cocktail (DHPG, histamine, carbachol; 10 µM each) to
hippocampal slices taken from littermate controls elicited robust increases in astrocyte intracellular Ca\(^{2+}\) (65% of 144 cells from 17 slices, 5 animals total; Figure 2.2A,B). In striking contrast, the GPCR cocktail failed to elicit Ca\(^{2+}\) increases in IP\(_3\)R2 KO hippocampal astrocytes (0% of 104 cells from 11 slices; 5 animals total; Figure 2.2A, B). Similar results were obtained in experiments using 100 µM ATP, with 80% of 40 astrocytes from littermate controls (4 slices total from 1 animal) and 0% of 38 astrocytes from IP\(_3\)R2 KOs (3 slices total from 1 animal) responding with Ca\(^{2+}\) increases (Figure 2.2B). In experiments where thapsigargin (2 µM) was applied as a control for Ca\(^{2+}\) increases, both littermate control (45% of 40 cells, 4 slices total from 1 mouse) and IP\(_3\)R2 KO (45% of 38 cells, 3 slices total from 1 mouse) astrocytes responded to thapsigargin with increases in intracellular Ca\(^{2+}\), indicating that the Ca\(^{2+}\) stores themselves were intact (Figure 2.2A, B).

Astrocytes have also been reported to exhibit spontaneous Ca\(^{2+}\) oscillations in the absence of neuronal activity (Nett et al., 2002). To determine if IP\(_3\)R2 is necessary for spontaneous Ca\(^{2+}\) oscillations, Ca\(^{2+}\) measurements were analyzed in astrocytes in the absence of agonist application. In bulk loaded slices from littermate controls, spontaneous Ca\(^{2+}\) increases were observed in 21% of 144 total astrocytes (17 slices from 5 animals), but were absent in IP\(_3\)R2 KO astrocytes (0% of 104 total cells, 11 slices from 5 animals). Taken together, these data indicate that astrocytes lacking IP\(_3\)R2 are incapable of releasing Ca\(^{2+}\) from internal stores either spontaneously or in response to agonists for G\(_q\)-linked GPCRs.
Neuronal Ca$^{2+}$ increases are intact in IP$_3$R2 KO CA1 pyramidal neurons

To address the question of a potential functional role of IP$_3$R2 in hippocampal neurons, we conducted Ca$^{2+}$ imaging experiments on CA1 pyramidal neurons loaded with Ca$^{2+}$ indicator via a patch pipette. Application of a G$_q$-linked GPCR agonist cocktail (in µM: 50 DHPG, 10 histamine, 10 carbachol) in 1 µM TTX to block action potentials elicited Ca$^{2+}$ transients in IP$_3$R2 KO neurons (Figure 2.3A, 6 cells from 6 slices, 5 animals total) not significantly different in amplitude (Figure 2.3B, left; $p > 0.05$) or duration (Figure 2.3B, right; $p > 0.05$) from those measured in littermate controls (8 cells from 8 slices, 5 animals total). These results indicate that lack of IP$_3$R2 does not significantly alter IP$_3$R-dependent Ca$^{2+}$ signaling in CA1 pyramidal neurons.

Lack of spontaneous Ca$^{2+}$ oscillations in astrocytes does not affect CA1 pyramidal neuron sEPSCs.

It has been reported that spontaneous and evoked astrocyte Ca$^{2+}$ elevations lead to gliotransmitter release which modulates basal neuronal excitatory and inhibitory synaptic activity via the activation of neuronal mGluRs and iGluRs (Hassinger et al., 1995; Araque et al., 1998b; Kang et al., 1998; Parri et al., 2001; Fiacco and McCarthy, 2004; Liu et al., 2004b; Liu et al., 2004a). The effect of blocking astrocyte Ca$^{2+}$ elevations and therefore Ca$^{2+}$-dependent gliotransmitter release on basal neuronal excitatory activity has not been thoroughly addressed. Therefore, we performed whole cell patch clamp experiments on CA1 pyramidal
neurons and found that basic neuronal properties such as resting membrane potential (Control: \(-61.7 \pm 1.1\) mV, IP\(_3\)R2 KO: \(-60.3 \pm 0.8\) mV; \(p > 0.05\)), membrane resistance (Control: \(274.5 \pm 26.2\) MOhms, IP\(_3\)R2 KO: \(249.1 \pm 15.5\) MOhms; \(p > 0.05\)) and membrane capacitance (Control: \(89.6 \pm 5.5\) pF; IP\(_3\)R2 KO: \(96.4 \pm 4.8\) pF; \(p > 0.05\)) were not significantly different between littermate control and IP\(_3\)R2 KO neurons (Control: 21 cells from 20 slices, 10 animals total; IP\(_3\)R2 KO: 22 cells from 17 slices, 10 animals total). In addition, there were no significant differences found between littermate control and IP\(_3\)R2 KO AMPA-R sEPSCs peak amplitude (\(p = 0.62\)), 10-90% rise time (\(p > 0.05\)), decay tau (\(p > 0.05\)), and event frequency (\(p > 0.05\)) (Figure 2.4B. Control: 15 cells from 14 slices, 9 animals total; IP\(_3\)R2 KO: 16 cells from 11 slices, 9 animals total). Additionally, no significant differences were found in NMDAR-mediated sEPSCs in peak amplitude (\(p > 0.05\)), 10-90% rise time (\(p > 0.05\)), decay tau (\(p > 0.05\)), and event frequency (\(p > 0.05\)) (Figure 2.4D. Control: 15 cells from 13 slices, 6 animals total; IP\(_3\)R2 KO: 9 cells from 8 slices, 3 animals total). Taken together, these results indicate that lack of IP\(_3\)R-dependent Ca\(^{2+}\) increases in astrocytes has no significant effect on either spontaneous AMPA-R or NMDAR-mediated excitatory synaptic currents in CA1 pyramidal neurons.

*Ambient glutamate of astrocyte origin is not released in a Ca\(^{2+}\)-dependent manner*

Ambient glutamate levels in the hippocampus have been the focus of several recent studies (Herman and Jahr, 2007; Le Meur et al., 2007). Findings in this area suggest that the majority of ambient glutamate present in the hippocampus is of glial
origin and might be released in a Ca$^{2+}$-independent manner (Jabaudon et al., 1999; Cavelier and Attwell, 2005). To address if ambient glutamate release occurs in an Ca$^{2+}$-independent manner, whole cell currents were recorded from CA1 pyramidal neurons held at +40mV in ACSF containing 1 $\mu$M TTX and 100 $\mu$M picrotoxin to isolate NMDAR-mediated currents. Similar to previous reports (Herman and Jahr, 2007; Le Meur et al., 2007), application of the NMDAR antagonist D-APV (50 $\mu$M) revealed a tonic NMDAR current of 33.7 ± 6.0 pA in littermate control neurons (6 cells from 6 slices, 4 animals total; Figure 2.5A, B). Recordings done in IP$_3$R2 KO neurons found a D-APV-sensitive current that was not significantly different from that found in littermate control neurons (35.7 ± 9.2 pA; $p > 0.05$; 5 cells from 5 slices, 3 animals total; Figure 2.5A, B).

To determine if removal of astrocytic Ca$^{2+}$ increases affects ambient glutamate accumulation during elevated extracellular glutamate, glutamate transporters were blocked using 100 $\mu$M TBOA. Application of TBOA caused a 7.0 ± 1.3 fold change in the tonic NMDAR current of littermate control neurons (6 cells from 6 slices, 4 animals total) and a similar fold change in KO neurons of 8.6 ± 1.1 (5 cells from 5 slices, 3 animals total; $p > 0.05$; Figure 2.5C, D). Overall, these data indicate that astrocyte Ca$^{2+}$ elevations do not play a significant role in regulating the ambient extracellular concentration of glutamate.
2.4. DISCUSSION

Astrocytes display both spontaneous and evoked intracellular Ca\textsuperscript{2+} increases using a variety of stimulation protocols (Montana et al., 2006). Astrocyte Ca\textsuperscript{2+} increases are due to the release of Ca\textsuperscript{2+} from internal stores upon activation of IP\textsubscript{3}Rs (Sheppard et al., 1997; Scemes, 2000). In this communication, we show that genetic disruption of IP\textsubscript{3}R2 abolishes both spontaneous and G\textsubscript{q}-linked GPCR agonist evoked IP\textsubscript{3}R-dependent Ca\textsuperscript{2+} increases in astrocytes. To our knowledge, the findings presented here are the first demonstration that astrocyte Ca\textsuperscript{2+} release in situ is functionally reliant on IP\textsubscript{3}R2. In contrast, IP\textsubscript{3}R-dependent Ca\textsuperscript{2+} increases in CA1 pyramidal neurons remains intact, indicating that IP\textsubscript{3}R2 is not necessary for neuronal IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} increases, or that IP\textsubscript{3}R2 may not be expressed by CA1 pyramidal neurons. This is supported by immunostaining data showing that IP\textsubscript{3}R2 is not expressed in neurons (Sharp et al., 1999; Hertle and Yeckel, 2007). It is somewhat surprising that in astrocytes, in which IP\textsubscript{3}R-dependent intracellular Ca\textsuperscript{2+} signals are thought to modulate an increasingly large number of key processes in brain (neuronal excitability, synaptic plasticity, and cerebrovascular control), that deletion of IP\textsubscript{3}R2 should result in the complete loss of Ca\textsuperscript{2+} activity without any apparent form of compensation. It is even more surprising that these mice: i) are not embryonic lethal, ii) do not show early mortality, and iii) do not exhibit any obvious histological or behavioral phenotype. They appear healthy, breed well and live normal lifespans. This is in stark contrast to the IP\textsubscript{3}R1 KO mouse model, which displays tonic-clonic seizures, ataxia and either die in utero or by weaning (P21) age (Matsumoto et al., 1996). Further behavioral testing of the IP\textsubscript{3}R2 KO mouse model
will provide a valuable insight into the role of astrocyte Ca²⁺-dependent signaling in specific animal behaviors, such as learning and memory.

Astrocytes have been implicated as a major source of ambient glutamate in the hippocampus (Jabaudon et al., 1999; Cavelier and Attwell, 2005; Herman and Jahr, 2007; Le Meur et al., 2007). In the present study, removal of astrocyte Ca²⁺ increases has no effect on the amplitude of the tonic NMDAR-mediated current activated by ambient glutamate. Furthermore, use of TBOA to block glutamate transporters revealed that the extent to which ambient glutamate accumulates during transporter block is unaffected by removal of IP₃R2 in astrocytes (Figure 2B). These findings are in agreement with the hypothesis that ambient glutamate release from astrocytes occurs in a Ca²⁺-independent manner, possibly through non-vesicular release mechanisms such as connexin hemichannels, P2X channels or anion channels (Cavelier and Attwell, 2005; Malarkey and Parpura, 2008).

A substantial literature has developed in the field of astrocyte biology concerning the role of Ca²⁺-dependent release of gliotransmitters such as ATP (which is converted to adenosine by ectonucleotidases) and glutamate on neuronal activity (see reviews by Carmignoto and Fellin, 2006; Fellin et al., 2006c; see reviews by Fiacco and McCarthy, 2006). These findings led to the development of the tripartite synapse model, in which astrocytes are active participants in synaptic transmission through IP₃R-mediated, Ca²⁺-dependent gliotransmitter release (Araque et al., 1999). While the majority of studies have focused on the outcome of pharmacologically evoking astrocyte Ca²⁺, very few studies have directly described the effect of blocking IP₃R-dependent Ca²⁺ release on basal excitatory neuronal
activity. Furthermore, it has been reported that spontaneous Ca\(^{2+}\) increases and subsequent glutamate release from astrocytes directly evokes NMDAR-mediated currents in neurons (Parri et al., 2001). Additionally, astrocyte Ca\(^{2+}\) increases have been associated with neuronal Ca\(^{2+}\) increases mediated by iGluRs (Hassinger et al., 1995; Pasti et al., 2001; Fellin et al., 2004).

A mathematical model incorporating data from numerous studies, including our own (Fiacco and McCarthy, 2004), on the role of astrocytes at the tripartite synapse predicts that astrocytes enhance synaptic release (Nadkarni and Jung, 2007). This is reflected by an increase in spontaneous postsynaptic events during and immediately following astrocyte Ca\(^{2+}\) elevations that trigger astrocytic release of glutamate compared to synapses lacking an associated astrocyte (Nadkarni and Jung, 2007). According to this model, a lack of astrocyte Ca\(^{2+}\) increases would produce a reduced event frequency, reflecting reduced synaptic release. It has also been reported that heterosynaptic depression due to Ca\(^{2+}\)-dependent ATP release from astrocytes suppresses glutamate release at CA3-CA1 synapses (Pascual et al., 2005). Based on these findings, it is reasonable to speculate that abolishment of Ca\(^{2+}\) increases in IP\(_3\)R2 KO astrocytes would lead to significant changes in basal excitatory neuronal activity and perhaps long term changes in brain activity and behavior. In recordings of sEPSCs from IP\(_3\)R2 KO and littermate control CA1 pyramidal neurons, we found no significant differences in peak amplitude, 10-90% rise time and decay tau of both AMPA-R and NMDAR-mediated synaptic currents. Furthermore, we find no change in the frequency of AMPA-R and NMDAR-mediated sEPSCs suggesting that lack of Ca\(^{2+}\) increases and Ca\(^{2+}\)-dependent gliotransmitter
release may not significantly affect baseline release probability from neuronal synaptic terminals.

The IP₃R2 KO mouse model offers a compliment to another mouse model developed in our laboratory that enables selective stimulation of G₉-GPCR signaling cascades in astrocytes (Fiacco et al., 2007). The findings presented here support our recent discovery using the MrgA1 transgenic mice that selective, widespread astrocyte Ca²⁺ elevations have no effect on baseline neuronal excitatory synaptic activity. It has been reported previously by a number of labs (including our own) that mechanical stimulation or uncaging Ca²⁺ or IP₃ in astrocytes leads to gliotransmitter release and changes in neuronal excitatory activity (Parpura and Haydon, 2000; Fiacco and McCarthy, 2004), neuronal inhibitory activity (Kang et al., 1998; Liu et al., 2004b; Liu et al., 2004a) and cerebrovascular tone (Zonta et al., 2003; Straub et al., 2006). While use of these pharmacological tools may elicit such responses, they may represent a non-physiological level of stimulation that does not occur in vivo and therefore does not accurately recapitulate endogenous IP₃ generating signaling pathways. There may be important regulatory mechanisms activated in GPCR signaling that work downstream of Ca²⁺ to inhibit vesicular release of glutamate by astrocytes. The IP₃R2 KO and MrgA1 mouse models together fully corroborate the concept that stimulation of astrocytic Gq-GPCRs and spontaneous astrocyte Ca²⁺ activity are not sufficient to cause vesicular release of glutamate from astrocytes, and that astrocytic IP₃R-mediated Ca²⁺ elevations are not necessary for normal neuronal excitatory synaptic activity in hippocampal CA1 pyramidal neurons.
The IP$_3$R2 mouse model also affords significant improvement over previously used techniques to examine the necessity of evoked IP$_3$-dependent Ca$^{2+}$ increases to changes in neuronal synaptic activity. Calcium chelators such as BAPTA or the bulk loadable BAPTA-AM have been used to block astrocyte Ca$^{2+}$ increases. There are technical issues with the use of BAPTA and Ca$^{2+}$ chelators that are eliminated by the use of the IP$_3$R2 KO mouse model. In the IP$_3$R2 KO, release of Ca$^{2+}$ from astrocyte internal stores is removed specifically, without causing a global change in the resting cytoplasmic Ca$^{2+}$ concentration or blocking Ca$^{2+}$ increases from other cellular sources.

In conclusion, the IP$_3$R2 KO mouse model represents a significant step forward in our ability to study the astrocyte Ca$^{2+}$ contribution to key physiological processes. Use of this model has the potential to clarify and further define the role of astrocytes in physiology and pathology without the use of pharmacological manipulations to block astrocyte Ca$^{2+}$ increases. This model can be used to identify Ca$^{2+}$-dependent and Ca$^{2+}$-independent mechanisms and their influence on astrocyte-neuronal communication, as well as re-evaluate the many important brain functions to which Ca$^{2+}$-dependent gliotransmitter release has been reported to play a significant role.
Figure 2.1 Histological analysis of IP$_3$R2 KO brains reveals no obvious abnormalities. Histological staining of brain sections taken from littermate control ($n = 3$) and IP$_3$R2 KO mice ($n = 3$). Six micron thick paraffin embedded sections were cut and stained with hematoxylin and eosin to visualize brain cytoarchitecture. No difference in the gross overall morphology or in the general cell layering was apparent between the IP$_3$R2 KO mice and littermate controls in any brain region; data from hippocampus, cortex and cerebellum are shown.
Figure 2.2 Knockout of IP₃R2 abolishes astrocyte GPCR-mediated Ca²⁺ increases.  

A. Representative Ca²⁺ traces from astrocytes of Calcium Green AM loaded hippocampal slices. Regions of interest were placed over the cell bodies of bulk loaded hippocampal astrocytes to measure Ca²⁺ increases in response to agonist application (upper panels). Application of ATP (100 µM) or a Gq-linked GPCR agonist cocktail (Ct: 10 µM DHPG, 10 µM histamine and 10 µM carbachol) elicited Ca²⁺ responses in astrocytes from littermate control but not IP₃R2 KO hippocampal slices. The arrows indicate the astrocyte Ca²⁺ traces shown in the lower panels. Thapsigargin (Tg, 2 µM) was used as a control and increased Ca²⁺ in astrocytes from littermate control and IP₃R2 Kos. Data presented as fold increases over baseline.

B. Percentage of astrocytes responding to application of ATP or the Gq-linked GPCR agonist cocktail from all experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>IP₃R2 KO</th>
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<tbody>
<tr>
<td>ATP</td>
<td>80.0% (32/40 cells)</td>
<td>0% (38/38 cells)</td>
</tr>
<tr>
<td>Cocktail (Ct)</td>
<td>65.0% (93/144 cells)</td>
<td>0% (0/104 cells)</td>
</tr>
<tr>
<td>Thapsigargin (Tg)</td>
<td>45.0% (18/40 cells)</td>
<td>45.0% (17/38 cells)</td>
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</table>
Figure 2.3 Lack of IP$_3$R2 does not affect neuronal Ca$^{2+}$ signaling. A. Representative Ca$^{2+}$ traces from CA1 pyramidal neurons patch clamped with internal solution containing Fluo-4 Ca$^{2+}$ indicator in response to application of a Gq-linked GPCR cocktail (50 µM DHPG, 10 µM histamine, 10 µM carbachol) in the presence of 1 µM TTX to block action potentials. B. Amplitude and duration of IP$_3$R-mediated Ca$^{2+}$ responses in CA1 pyramidal neurons (Control n = 8; IP$_3$R2 KO n = 6). There were no significant differences for amplitude (left panel; p > 0.05) or duration (right panel; p > 0.05). Error bars indicate SEM.
Figure 2.4 Spontaneous EPSCs from CA1 pyramidal neurons are unchanged in IP$_3$R2 KO mice. A. Representative AMPA-R sEPSC traces from littermate control ($n = 15$) and IP$_3$R2 KO ($n = 16$) CA1 pyramidal neurons. B. AMPA-R sEPSC peak amplitude ($p > 0.05$), 10-90% rise times ($p > 0.05$), decay tau ($p > 0.05$), and event frequency ($p > 0.05$) were not significantly different between littermate control and IP$_3$R2 KO CA1 pyramidal neurons as determined by Student's t-test. C. Representative NMDAR sEPSC traces from littermate control ($n = 15$) and IP$_3$R2 KO ($n = 9$) CA1 pyramidal neurons. D. NMDAR sEPSC peak amplitude ($p > 0.05$), 10-90% rise times ($p > 0.05$), decay tau ($p > 0.05$), and event frequency ($p > 0.05$) were not significantly different between littermate control and IP$_3$R2 KO CA1 pyramidal neurons as determined by Student's t-test. Error bars indicate SEM.
Figure 2.5. Ambient glutamate is unaffected by the lack of Ca\(^{2+}\) increases in astrocytes.  

A. Bath application of 50 µM D-AP5 blocked a tonic NMDAR current in CA1 pyramidal neurons held at +40mV in ACSF from both littermate control and IP\(_3\)R2 KOs.  

B. The amplitude of the D-AP5 sensitive NMDAR current was not significantly different (\(p > 0.05\)) in IP\(_3\)R2 KO neurons (\(n = 5\)) versus littermate controls (\(n = 6\)).  

C. Representative traces showing that bath application of 100 µM TBOA induced a large fold increase of the tonic current in CA1 pyramidal neurons held at +40mV from both littermate control and IP\(_3\)R2 KOs.  

D. The fold increase over baseline induced by TBOA is not significantly different (\(p > 0.05\)) between IP\(_3\)R2 (\(n = 5\) cells) and littermate controls (\(n = 6\) cells). Error bars indicate SEM.
Chapter III: Gq-GPCR-mediated, IP$_3$R-dependent Ca$^{2+}$ increases in astrocytes does not affect CA3 pyramidal neuron excitatory synaptic activity.

3.1. INTRODUCTION

The modulation of neuronal synaptic activity by astrocytes has become an increasingly investigated area of neurophysiology. The hippocampus has been used as a model system for the investigation of astrocyte-derived neuromodulation, in particular at the Schaffer collateral – CA1 pyramidal neuron (SC-CA1) synapse (Fellin et al., 2004; Pascual et al., 2005; Fiacco et al., 2007; Perea and Araque, 2007). Numerous reports show that astrocytes can affect a wide range of synaptic activity of various scales, from synaptic currents at single synapses to the formation of long term potentiation (LTP) on a network-wide scale. At the synaptic scale astrocytes enhance the efficacy of single synapses of CA1 pyramidal neurons in response to stimulation and modulate the function of inhibitory synapses by targeting both metabotropic (mGluR) and ionotropic (iGluRs) glutamate receptors (Kang et al., 1998; Liu et al., 2004a; Perea and Araque, 2007). On the network level astrocytes affect heterosynaptic depression through the release of ATP/adenosine and the
formation of LTP through the release of D-serine (Pascual et al., 2005; Serrano et al., 2006; Henneberger et al., 2010).

Astrocytes are theorized to modulate synaptic activity through the release of gliotransmitters including glutamate, ATP (which is broken down to adenosine) and D-serine. These transmitters are released from astrocytes in a Ca\(^{2+}\)-dependent manner in response to either electrical or pharmacological stimulation (Agulhon et al., 2008). The primary mechanism for the elevation of intracellular Ca\(^{2+}\) levels in astrocytes is through the Gq-GPCR-PLC-IP\(_3\) pathway (Fiacco and McCarthy, 2006; Haydon and Carmignoto, 2006). However, recent advances in genetic mouse models have provided evidence that the Gq-GPCR-PLC-IP\(_3\) pathway may not be involved in the release of gliotransmitters under physiological conditions (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010). Previous studies using selective activation the MrgA1 mouse model did not result in modulation of CA1 pyramidal neuron synaptic activity or the formation of LTP (Fiacco et al., 2007; Agulhon et al., 2010). We presented evidence in Chapter II that lack of IP\(_3\)R-mediated Ca\(^{2+}\) increases in the IP\(_3\)R2 KO does not lead to changes in basal excitatory postsynaptic activity of CA1 pyramidal neurons as predicted by the gliotransmission literature (Petravicz et al., 2008). Additionally, the IP\(_3\)R2 cKO mice had no deficit in the induction of LTP (Agulhon et al., 2010). These data call into question the functional relevance of IP\(_3\)R-mediated Ca\(^{2+}\) increases in gliotransmission.

While the SC-CA1 synapse has been widely studied in regards to gliotransmission, the mossy fiber-CA3 pyramidal neuron (MF-CA3) synapse remains
a relatively unexplored. The MF-CA3 synapse is marked by a low release probability due largely to the activity of adenosine A1 receptors (A1Rs) on voltage gated Ca\(^{2+}\) channels on the MF presynaptic terminals (Moore et al., 2003; Nicoll and Schmitz, 2005). Mossy fiber terminals are also marked by high expression of metabotropic glutamate group II receptors (mGluR2 and 3) that serve as autoreceptors to diminish presynaptic release (Vogt and Nicoll, 1999). Postsynaptically, the MF-CA3 synapse contains high levels of iGluRs (primarily AMPA and KA receptors) that are responsible for the majority of excitatory post synaptic currents (McBain and Dingledine, 1992; Cossart et al., 2002). It has been shown that a large percentage of MF-CA3 miniature excitatory post synaptic currents are AMPA/KAR-mediated (McBain and Dingledine, 1992). Astrocytes release gliotransmitters (primarily glutamate and ATP/adenosine) capable of activating several of the receptor types involved in regulating MF-CA3 synaptic transmission, specifically quantal release measured by mEPSCs. The MF-CA3 synapse is a potential model for measuring gliotransmission, given the well documented regulation of MF-CA3 transmission by neurotransmitters thought to be released by astrocytes. However, to date there are no direct investigations concerning the role of gliotransmission in activating pre- or postsynaptic receptors at the MF-CA3 synapse to modulate synaptic transmission.

In this chapter, we report that selective increase or blockade of astrocyte intracellular Ca\(^{2+}\) levels has no effect on CA3 pyramidal neuron mEPSC synaptic currents. Activation of astrocytic Gq GCPRs, either with endogenous ligand or with the MrgA1 receptor ligand FMRF, resulted in Ca\(^{2+}\) increases in the majority of CA3
astrocytes. However no effect was found on peak amplitude, decay tau, 10-90% rise time or frequency of CA3 pyramidal neuron mEPSCs in response to application of ligand shown to cause increases in astrocyte Ca\textsuperscript{2+}. Our results indicate that astrocyte IP\textsubscript{3}R-mediated, Ca\textsuperscript{2+}-dependent release of gliotransmitters is not involved in the modulation of CA3 pyramidal neuron mEPSCs.
3.2. METHODS

**Generation of IP$_3$R2 cKO Mice**

Floxed IP$_3$R2 mice were generated as previously described in Chapter 2 and by Li et al (2005). To generate IP3R2 conditional knockout (cKO) mice, IP$_3$R2$^{flox/flox}$ mice were bred to mice expressing the Cre-recombinase protein under the control of a fragment of the glial fibrillary acidic protein (GFAP) promoter (Casper and McCarthy, 2006). Mice double heterozygous for the floxed (IP$_3$R2$^{flox/flox}$) and Cre-recombinase alleles were interbred to IP$_3$R2$^{flox/flox}$ mice to generate conditional knockout mice homozygous for the floxed IP$_3$R2 allele and heterozygous for Cre-recombinase (IP$_3$R2 cKO). Mice homozygous for IP$_3$R2$^{flox/flox}$ but null for the Cre-recombinase allele were designated as littermate controls (Control or WT). Mice were genotyped by PCR analysis using genomic DNA and primers specific to Cre-recombinase and the floxed IP$_3$R2 allele.

**Histology**

For cytoarchitectural analysis littermate control and IP$_3$R2 cKO brains were fixed with formalin and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin to stain nuclei and cell bodies. For the analysis of neuronal terminals in the CA3 region of the hippocampus, littermate control and IP$_3$R2 cKO brains were fixed with sulfide perfusate and postfixed with 10% formalin and embedded sagitally in paraffin. All brains were sectioned at 6-10 $\mu$M thickness with a Leica (Nussloch, Germany) microtome from p30 littermate controls and
sections were imaged using a Zeiss (Oberkochen, Germany) Axioscope light microscope.

*Dendritic Spine Density*

Littermate control and IP₃R2 cKO animals were stained using a Rapid GolgiStain Kit from FD Biosciences (Ijamsville, MD). Briefly, brains were rapidly removed from deeply anesthetized mice, rinsed briefly in distilled water, and immersed in impregnation solution for one week at room temperature. After incubation with cyroprotectant solution, coronal sections through the hippocampus (100-120 µM thick) were cut on a Leica VT1000S vibrating blade microtome, mounted on slides and sealed with Permount (Thermo Fisher Scientific) and imaged using a Zeiss (Oberkochen, Germany) Axioscope light microscope using a 40x lens and a Dage MTI XML Excel digital camera. Spine numbers on primary and secondary dendrites in the stratum radiatum of CA3 pyramidal neurons were counted and spine density was calculated by dividing the number of spines by the dendritic length to determine spines per µm length.

*Preparation of Acute Hippocampal Slices*

All procedures followed the guidelines of the IACUC of UNC-Chapel Hill. Littermate control and IP₃R2 cKO mice 16 to 22 days old (P16-22) were anesthetized by isoflurane inhalation. The brains were rapidly removed after
decapitation and submerged into 4°C slicing buffer containing the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 5 MgCl$_2$, and 1 CaCl$_2$ and bubbled with 95% O$_2$ and 5% CO$_2$. Brains were cut sagittally at a thickness of 300 µm on a Vibratome (Bannockburn, IL) vibratory microtome. During sectioning brains were kept submerged in 4°C oxygenated slicing buffer. Hippocampi were dissected out of each brain slice and incubated 45 min in artificial cerebral spinal fluid (ACSF) warmed to 35-37°C and bubbled continuously with 95% O$_2$ and 5% CO$_2$. The ACSF contained the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 2.5 CaCl$_2$, and 1.5 MgCl$_2$.

Calcium Imaging

Hippocampal slices were prepared as described above except the brains were sectioned in a modified slicing buffer containing the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 5 MgCl$_2$, and 1 CaCl$_2$ and bubbled with 95% O$_2$ and 5% CO$_2$. Hippocampal slices were incubated at 35-37°C for 20 min in slicing buffer containing 1µM SR101 to preferentially load astrocytes (Nimmerjahn et al., 2004). Hippocampal slices were then transferred for 10 min to warm (35-37°C) ACSF containing the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 2.5 MgCl$_2$, and 1.5 CaCl$_2$ and bubbled with 95% O$_2$ and 5% CO$_2$. Hippocampal slices were then removed from the incubator and kept at room temperature for the duration of the experiment. The calcium indicator Oregon Green BAPTA-AM (OGB-1AM) was suspended in 100µl of a modified bolus loading
ACSF containing 20% pluronic acid (final DMSO concentration 0.4%). The ACSF contained the following in mM: 150 NaCl, 2.5 KCl and 10 HEPES with the pH adjusted to 7.3-7.5 with 1M NaOH.

Hippocampal slices were placed in a perfusion chamber with a constant flow of oxygenated normal ACSF. Pipettes (1-2MΩ resistance) filled with the OGB-1AM containing bolus loading ACSF were lowered to the surface of the slices and backpressure applied. The pipette was then lowered 40 µm into the hippocampal stratum to the pyramidal cell layer of the CA3 region. OGB-1AM was injected for 2-3 minutes (based on the pipette resistance) and then lowered a further 35 µm deeper into the slice and injected for an additional 2-3 minutes. The pipette was then removed and the slice transferred to room temperature (25°C) oxygenated ACSF and allowed to recover for a minimum of 45 min prior to imaging.

Astrocyte calcium increases were recorded using a two photon imaging system (Coherent Chameleon Ultra, Coherent Inc, Santa Clara, CA). Astrocytes were identified by SR101 loading and regions of interest were drawn around the SR101 positive cell bodies. Increases in average fluorescence in regions of interest indicate increase in Ca\(^{2+}\) concentration. Fold increase over baseline was calculated for each trace and reported as \(\Delta F/F_0\).
Electrophysiology

For recording of AMPA/KA receptor mEPSCs, whole cell patch clamp of CA3 pyramidal neurons was performed using pipettes (3.0-4.0MΩ resistance) filled with an internal solution containing in mM: 100 Cs-gluconate, 8 NaCl, 0.6 EGTA, 5 MgCl2, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, 0.1 spermine, and 1 N-(2,6-dimethylphenyl carbamoylmethyl)-triethylammonium bromide (QX-314), pH 7.2-7.3. Neurons were identified by their location and morphology and voltage clamped at a membrane potential of -60mV. Neurons with an access resistance (Ra) of over 40MΩ were used for mEPSCs recordings. Any neurons that changed their Ra more than 20% over the course of the experiment were not included in the analysis. Neurons were recorded at room temperature (25°C) in ACSF containing 1µM TTX, 10µM bicuculline and 50µM APV.

Data Collection and Analysis

Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Traces were analyzed for EPSCs in Clampfit 10.2 software (Axon Instruments) using a template constructed from 4-6 mEPSCs intrinsic to each recording. The event statistics were taken for each individual event and then averaged. The averaged event statistics from each cell were then averaged together, normalized to the baseline and reported as mean ± SEM. For calcium imaging, data were collected and analyzed using Olympus Fluoview software (Olympus America Inc, Center Valley, PA). Statistical analysis of
electrophysiological recordings was performed using Statview v5.0 software. A repeated measure ANOVA was used to analyze the normalized whole cell mEPSC recordings. Student’s t-test was used to analyze the basal mEPSC frequency and dendritic spine density.

Reagents

D-AP5, tetrodotoxin (TTX), CNQX, DHPG, histamine, carbachol, and spermine were obtained from Tocris Biosciences (UK). Oregon Green BAPTA-AM was obtained from Invitrogen (Carlsbad, CA). SR101, FMRF, QX-314, bicuculline, and other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).
3.3. RESULTS

_Histological analysis of IP$_3$R2 cKO mouse brains._

It has been shown that IP$_3$R based signaling in radial glial cells is important for the proliferation of neurons in the neocortex (Weissman et al., 2004). Additionally, the GFAP-Cre expresses in a developmentally timed manner that could have an effect on the proper formation of brain structures (Casper and McCarthy, 2006). We performed histological staining of paraffin embedded section from adult IP$_3$R2 cKO and littermate control mice to determine if there were any obvious abnormalities in brain cytoarchitecture indicative of improper proliferation during development. Hematoxylin and eosin staining of P30 littermate control (n = 4) and IP$_3$R2 cKO (n = 4) brains revealed no significant abnormalities in cytoarchitecture (Figure 3.1, A-D). This is in agreement with our previous study in the full IP$_3$R2 KO, in which the lack of IP$_3$R2 during development was shown to not affect gross brain cytoarchitecture (see Figure 2.1, Chapter II).

_Dendritic spine density of CA3 hippocampal pyramidal neurons_

Astrocytes extensively interact with dendritic spines in both organotypic hippocampal slice cultures and _in vivo_ and can promote synaptogenesis (Christopherson et al., 2005; Haber et al., 2006). Changes in dendritic spine density can also influence synaptic activity and signal integration in neurons (Tsay and Yuste, 2004). We performed Golgi staining of littermate control and IP$_3$R2 cKO brains to determine if there was a change in CA3 pyramidal neuron dendritic spine density.
density. Spines from primary and secondary dendrites of CA3 pyramidal neurons were counted and the spine density per dendritic shaft length was determined. There was no significant difference found in the density of dendritic spines between IP₃R2 cKO (0.37 ± 0.02 spines per 1µm; n = 487 spines. Figure 3.2) and littermate controls (0.32 ± 0.01 spines per µm; n = 468 spines; p > 0.05. Figure 3.2). This provides anatomical data that is in agreement with our findings that lack of IP₃R2-mediated Ca²⁺ increases has not affected the properties of CA3 pyramidal neuron mEPSCs.

*IP₃R2 cKO hippocampal astrocytes lack Gq-GPCR-mediated Ca²⁺ increases.*

Astrocytes respond to a wide variety of Gq-GPCRs with Ca²⁺ increases, both through agonist application and in response to electrical stimulation (Porter and McCarthy, 1996; Verkhratsky and Kettenmann, 1996). We previously reported that lack of IP₃R2 in astrocytes completely abolishes both spontaneous and agonist evoked Ca²⁺ increases in hippocampal astrocytes ((Petravicz et al., 2008) Chapter II). To examine the efficiency of the GFAP-Cre system in recombining the floxed IP₃R2 allele, we performed Ca²⁺ imaging on bolus loaded hippocampal slices from littermate control and IP₃R2 cKO mice. Hippocampal slices were loaded with SR101 to selectively label astrocytes (Nimmerjahn et al., 2004). Calcium increases were recorded from SR101 positive astrocytes in the stratum radiatum of the CA3 region of the hippocampus. Bath application of a Gq-GPCR agonist cocktail (DHPG, histamine, carbachol; 10 µM each) to hippocampal slices taken from littermate
controls elicited robust Ca^{2+} increases in the majority of SR101-positive astrocytes (92.8% of 69 cells from 10 slices, 5 animals total; Figure 3.3B, upper left traces). The majority of astrocytes also responded to bath application of agonists to Gq-GPCR endothelin receptors 1 and 3 (ET1 and ET3, 10 nM each) with Ca^{2+} increases (85.7% of 42 cells from 6 slices, 4 animals total; Figure 3.3B, upper right traces).

In contrast, bath application of the same Gq-GPCR agonist cocktail or ET1/ET3 to hippocampal slices from IP_{3}R2 cKO mice was incapable of eliciting a Ca^{2+} response in SR101 positive astrocytes (0% of 40 cells, 6 slices from 4 animals for Gq-GPCR agonist cocktails; 0% of 39 cells, 6 slices from 4 animals for ET1/ET3 agonist; Figure 3.3B, lower traces). This is in agreement with studies in Chapter II using the full IP_{3}R2 KO mouse model indicating that IP_{3}R2 is a key element in the Gq-GPCR pathway in astrocytes. Furthermore, these data indicate that GFAP-Cre is effective at recombining the floxed IP_{3}R2 allele in the hippocampus.

*Lack of Gq-GPCR evoked IP_{3}R2 mediated Ca^{2+} increases in astrocytes does not affect CA3 pyramidal neuron mEPSCs.*

It has been reported that Gq-GPCR evoked, IP_{3}R2-mediated Ca^{2+} increases in astrocytes trigger the release of gliotransmitters to modulate both excitatory and inhibitory synaptic activity (Liu et al., 2004b; Jourdain et al., 2007). We performed whole cell voltage clamp recordings of mEPSCs from CA3 pyramidal neurons to determine if lack of spontaneous IP_{3}R2-mediated Ca^{2+} increases affected the basal frequency of mEPSCs and if application of endothelin receptor agonists (ET1/3)
shown to increase astrocyte Ca\textsuperscript{2+} cytosolic levels triggers the release of gliotransmitters to modulate mEPSCs parameters. CA3 pyramidal neurons were identified by their location and morphology. Whole cell recordings of mEPSCs were done in the presence of the sodium channel blocker TTX (1\textmu M), the GABAA receptor antagonist bicuculline (10 \textmu M), and the NMDA receptor antagonist D-APV (50 \textmu M) to isolate AMPA/KA mediated currents.

Basal mEPSCs were recorded for 3 min prior to a 2 min application of ET1/3 (20 nM), followed by a 9 min ACSF wash period and a 3 min application of CNQX (50 \textmu M) to block the AMPA/KA mEPSCs. No significant differences were found in the basal mEPSC frequency between CA3 pyramidal neurons from littermate controls and IP\textsubscript{3}R2 cKO (0.45 ± 0.11 Hz for controls; 0.57 ± 0.19 Hz for IP\textsubscript{3}R2 cKO; p > 0.05; Figure 3.4B insert). Additionally, there was no effect upon application of Gq-GPCR agonists to endothelin receptors ET\textsubscript{A} and ET\textsubscript{B} either during the 2 min application or during the 9 min ACSF wash period in frequency (p >0.05), peak amplitude (p > 0.05), decay tau (p > 0.05) or 10-90% rise time (p > 0.05) of the mEPSCs during any of the condition analyzed (Figure 3.4B). Application of CNQX blocked all occurrences of mEPSCs, confirming that the events recorded were mediated by activation of AMPA/KA receptors. As a positive control, application of the A1R antagonist 8-Cyclopentyltheophylline (CPT, 1 \textmu M) was used to detect changes to A1R mediated inhibition. CPT induced a 286.5 ± 46.2% (n = 3) increase in CA3 pyramidal neuron mEPSC frequency. Taken together, these results indicate that lack of Gq-GPCR, IP\textsubscript{3}R2-mediated Ca\textsuperscript{2+} increases in astrocytes has no
significant effect on miniature excitatory post synaptic current of CA3 pyramidal neurons.

*Selective activation of astrocyte Ca$^{2+}$ increases in the MrgA1 mouse model does not affect CA3 pyramidal neuron mEPSCs.*

To selectively activate Ca$^{2+}$ increases in astrocytes of the CA3 pyramidal layer in the hippocampus, we used the MrgA1 mouse model. The MrgA1 mouse model enables selective activation of Ca$^{2+}$ increases in GFAP+-cells in situ in CA1 astrocytes and SVZ GFAP+ cells (Fiacco et al., 2007; Platel et al., 2010). Ca$^{2+}$ imaging was performed on acute hippocampal slices from control and MrgA1+ mice to determine if CA3 astrocytes respond to bath application of the MrgA1+ synthetic agonist FMRF. Hippocampal slices were loaded with SR101 to identify astrocytes. Bath application of FMRF (15$\mu$M) elicited robust Ca$^{2+}$ increases in MrgA1+ astrocytes (87.8% of 33 cells; 3 slices from 2 animals) but not in littermate controls (0% of 28 cells; 3 slices from 2 animals, Figure 2.5C). Bath application of a Gq-GPCR agonist cocktail (DHPG, histamine, carbachol; 10 $\mu$M each) elicited Ca$^{2+}$ increase in both the MrgA1+ (87.8% of 33 cells; 3 slices from 2 animals) and littermate controls (78.5% of 28 cells; 3 slices from 2 animals, Figure 2.5C). All SR101 positive astrocytes from MrgA1+ hippocampal slices that responded to FMRF responded to the agonist cocktail. These results indicate FMRF application activates a similar percentage of MrgA1+ CA3 astrocytes as a cocktail of endogenous Gq-GPCR agonists.
Whole cell recordings of mEPSCs were done in the presence of the sodium channel blocker TTX (1 µM), the GABA$_A$ receptor antagonist bicuculline (10 µM), and the NMDA receptor antagonist D-APV (50 µM) to isolate AMPA/KA mediated currents resulting from quantal release from both MF and A/C synapses. Basal mEPSCs were recorded for 3 min prior to a 3 min application of FMRF (15 µM), followed by a 9 min ACSF wash period and a 3 min application of CNQX (50 µM) to block the AMPA/KA mEPSCs. No significant differences were found in the frequency ($p > 0.05$), peak amplitude ($p > 0.05$), decay tau ($p > 0.05$) or 10-90% rise time ($p > 0.05$) of the mEPSCs during any of the conditions analyzed (Figure 3.6B). Taken together, these results indicate that selective activation of the MrgA1+ receptor to increase Ca$^{2+}$ in hippocampal CA3 astrocytes does not trigger the release of gliotransmitters to modulate miniature excitatory post synaptic currents.
3.4. DISCUSSION

There is increasing evidence that astrocytes actively modulate neuronal synaptic activity; however the mechanisms and conditions are not clearly understood. It has been established that astrocytes are capable of responding to neuronal activity with increases in intracellular Ca\(^{2+}\) through activation of Gq-GPCRs and downstream activation of PLC and IP\(_3\)Rs (Fiacco and McCarthy, 2006). This has led to numerous published studies and reviews arguing that Gq-GPCR-mediated, IP\(_3\)R-dependent increases in intracellular Ca\(^{2+}\) trigger the release of gliotransmitters from astrocytes (Volterra and Steinhauser, 2004; Zhang and Haydon, 2005; Halassa et al., 2009a; Hamilton and Attwell, 2010). The SC-CA1 synapse of the hippocampus has been widely used a model system for the study of gliotransmitter-dependent neuronal modulation. In the hippocampus, astrocytes are capable of modulating SC-CA1 excitatory synaptic activity by the release of glutamate (Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2007), ATP/adenosine (Pascual et al., 2005; Serrano et al., 2006), and D-serine (Oliet and Mothet, 2009; Henneberger et al., 2010). Further, modulation by gliotransmitters occurs from the single synapse level up to the network level (Pascual et al., 2005; Perea and Araque, 2007). Astrocytes have also been reported not only to modulate excitatory synaptic activity, but inhibitory activity as well through the Ca\(^{2+}\)-dependent release of gliotransmitters (Kang et al., 1998; Liu et al., 2004a). Reports of astrocyte derived modulation of neuronal activity vary between pre- or postsynaptic effects on neurons, with glutamate and ATP/adenosine positively or negatively regulating release probability and driving iGluR currents (Agulhon et al., 2008).
The SC-CA1 synapse represents only one of the major synapses present in the hippocampus. Equally critical is the synaptic connections between the mossy fiber axons of dentate gyrus granule cells and CA3 pyramidal neurons. These specialized synapses are markedly different from SC-CA1 synapses in both structure and release probabilities (Carnevale et al., 1997; Nicoll and Schmitz, 2005; Lauri et al., 2007). Release probability is low at MF-CA3 synapses, resulting in enhanced paired pulse facilitation and frequency facilitation not found at SC-CA1 synapses (Salin et al., 1996). This is due to tonic presynaptic inhibition by adenosine A1 receptors and to lesser degree group II metabotropic glutamate receptors (Vogt and Nicoll, 1999; Moore et al., 2003). Under certain condition, astrocytes release glutamate and ATP/adenosine to target A$_1$Rs and mGluRs on presynaptic terminals at SC-CA1 synapses (Fiacco and McCarthy, 2004; Pascual et al., 2005; Perea and Araque, 2007). This provides a foundation for investigating if astrocytes are actively participating in the regulation of CA3 pyramidal neuron synaptic activity. In the present study, we have employed two different genetic mouse models designed to evaluate the role of astrocytic IP$_3$R-mediated Ca$^{2+}$ increases. We present the novel finding that astrocyte IP$_3$R-mediated Ca$^{2+}$ increases do not affect CA3 pyramidal neuron synaptic activity measured by mEPSCs.

Calcium increases in radial glia have are critical to neurogenesis during development (Weissman et al., 2004) and astrocytes can regulate the formation and maintenance of dendritic spines in the brain (Ullian et al., 2001; Christopherson et al., 2005). Histological staining of IP$_3$R2 cKO brains revealed no significant
differences in cytoarchitecture, which is in agreement with our previous findings in
the full IP₃R2 KO ((Petravicz et al., 2008), Chapter II). Golgi staining of CA3
pyramidal neurons revealed that spine density of primary and secondary dendrites is unaffected by lack of IP₃R-mediated Ca²⁺ increases in astrocytes. This novel finding indicates that astrocytes may not regulate spine formation or maintenance in an IP₃R-mediated, Ca²⁺-dependent manner. Further experiments are required fully investigate any changes that may not be reflected by dendritic spine density.

To determine the role of IP₃R2 in Gq-GPCR mediated increases in Ca²⁺, IP₃R2 cKO mice were evaluated using two-photon Ca²⁺ imaging. Astrocytes from the CA3 region of IP₃R2 cKO hippocampi were found to be incapable of responding to Gq-GPCR agonist application with intracellular Ca²⁺ increases. This data is in agreement with our previous finding that IP₃R2 is a critical component to the Gq-GPCR-PLC-IP₃ pathway thought to underlie gliotransmission (Chapter II, Petravicz et al., 2008). Additionally, these data illustrate that GFAP-Cre is highly efficient at excising the floxed portion of the IP₃R2 gene and present advancement in the IP₃R2 genetic mouse model by restricting the knockout to the central nervous system.

Activation of A₁Rs by adenosine and group II mGluRs by glutamate regulate synaptic transmission at the MF-CA3 synapse (Vogt and Nicoll, 1999; Moore et al., 2003). Electrophysiological recording of CA3 pyramidal neuron mEPSCs were conducted to evaluate how lack of IP₃R-mediated Ca²⁺ responses may affect synaptic transmission. No change was found in mEPSC frequency between IP₃R2 cKO and littermate control, indicating that lack of spontaneous or Gq-GPCR evoked Ca²⁺ increase in astrocytes has not affected basal CA3 pyramidal neuron excitatory
activity. Further, application of a Gq-GPCR agonist failed to elicit changes to any of the mEPSC parameters measured either during drug application or during the subsequent wash period. Therefore, lack of IP$_3$R2 Ca$^{2+}$ increases in astrocytes in response to Gq-GPCR drug application does not affect CA3 pyramidal neuron excitatory synaptic activity measured by mEPSCs.

As a correlate to the IP$_3$R2 cKO model, the MrgA1 mouse model was used in near identical experiments. Calcium imaging of CA3 astrocytes showed that application of the synthetic MrgA1 ligand FMRF resulted in Ca$^{2+}$ increases in the majority of astrocytes. These data support the use of the MrgA1 mouse model for studies of astrocyte involvement in the modulation of CA3 pyramidal neuron synaptic activity. Analysis of CA3 pyramidal neuron mEPSCs from IP$_3$R2 cKO hippocampi revealed no significant changes in mEPSC parameters upon application of FMRF to stimulate MrgA1 receptors on CA3 astrocytes. Taken together, the IP$_3$R2 cKO and MrgA1 data indicate that astrocyte IP$_3$R-mediated Ca$^{2+}$ increases are not involved in the modulation of CA3 pyramidal neuron excitatory synaptic activity measured by mEPSCs. Further experiments using these mouse models to explore potential changes to paired pulse ratio and frequency facilitation will be valuable to determining if astrocytes may be modulating more intensive modes of evoked release at mossy fiber-CA3 pyramidal neuron synapses.

A potential role for astrocytes at this synapse may be inferred from ultrastructural studies. Rollenhagen et al (2007) recently showed that astrocyte processes ensheathe the majority of the surface area of the mossy fiber bouton and their postsynaptic targets, but that the astrocyte processes do not come in contact
with the majority of active zones. Based on this structural arrangement, astrocytes may not be in a position to affect the majority of active zones made by the mossy fiber bouton by either the release of gliotransmitters or reuptake of neurotransmitters from the synaptic cleft. However, as noted in Rollenhagen et al (2007), this structural arrangement may facilitate synaptic cross-talk between active zones in the mossy fiber bouton and contribute to their efficacy. Studies involving changes to astrocyte process coverage of mossy fiber boutons may provide insight into modulation of CA3 pyramidal neuron synaptic activity. There is also potential for this form of regulation to be involved the generation or modulation of pathological states of CA3 pyramidal neuron activity such as epilepsy.

The IP3R2 cKO mouse model presented in this chapter represents advancement over the model described in Petravicz et al (2008) and Chapter II. The use of the GFAP-Cre recombinase limits the deletion of IP3R2 to the CNS, reducing peripheral effects. This newer version of the IP3R2 KO mouse model is ideal for further studies; in particular behavioral experiments that will provide valuable insight in determining brain regions where astrocyte-neuronal communication may be playing a critical role. In conclusion, presynaptic release at the MF-CA synapse is known to be heavily regulated by neurotransmitter release and cross-talk between active zones. Astrocytes ensheathe the MF-CA synapse, however no evidence was found for Ca2+-dependent release of gliotransmitters to modulate synaptic transmission.
3.5. Figures and Tables

**Figure 3.1** Histological analysis of IP$_3$R2 cKO brains reveals no obvious abnormalities. Histological staining of brain sections taken from littermate control ($n = 4$) and IP$_3$R2 cKO mice ($n = 4$). Six micron thick paraffin embedded sections were cut and stained with hematoxylin and eosin to visualize brain cytoarchitecture. No difference in the gross overall morphology or in the general cell layering was apparent between the IP$_3$R2 cKO mice and littermate controls in any brain region; data from hippocampus, cortex (A, C) and cerebellum (B, D) are shown.
Figure 3.2. Dendritic spine density is not affected in the IP$_3$R2 cKO. Golgi staining of brains from littermate control (IP$_3$R2 WT, n = 3) and IP$_3$R2 cKO (n = 3) animals. Coronal sections 100-120 µm thick were cut from Golgi impregnated brains to examine hippocampal CA3 pyramidal neuron spine density. A. Primary and secondary dendrites from WT (28 dendrites from 15 neurons; 468 spines total) and an IP$_3$R2 cKO (28 dendrites from 15 neurons; 487 spines total) were imaged and the spine density calculated. Representative dendrites are shown. B. There was no significant difference found in the spine density between WT and IP$_3$R2 cKO ($p > 0.05$).
Figure 3.3. Conditional knockout of IP$_3$R2 abolishes Gq-GPCR elicited, IP$_3$R-mediated Ca$^{2+}$ increases in CA3 astrocytes. **A.** Images of hippocampal slices from littermate control (IP$_3$R2 WT) and IP$_3$R2 cKO brains. Slices were bulkloaded with SR101 (left panels) and pressure loaded with OGB-1AM (middle panels). The right panel shows co-localization of the SR101 and OGB-1AM in astrocytes simultaneously imaged for both. **B.** Bath application of either a Gq-GPCR agonist cocktail (DHPG, histamine, carbachol; 10µM each, left traces) or agonist for the endothelin receptors (ET1/ET3; 10nM each, right traces) failed to elicit increases in astrocyte Ca$^{2+}$ in IP$_3$R2 cKO astrocytes (bottom traces). Both the agonist cocktail
and ET1/ET3 elicited robust Ca\textsuperscript{2+} increases in IP\textsubscript{3}R2 WT astrocytes. Three representative traces are shown for IP\textsubscript{3}R2 WT and IP\textsubscript{3}R2 cKO for each condition. **C.** Table quantifying percentage of SR101 positive astrocytes that responded to application of agonist cocktail or ET1/ET3 from all experiments.
Figure 3.4. Lack of Gq-GPCR evoked IP$_3$R-mediated Ca$^{2+}$ increases in astrocytes does not affect mEPSC parameters. **A.** Representative traces of CA3 pyramidal neuron mEPSCs recorded from WT and IP$_3$R2 cKO hippocampal slices. CA3 pyramidal neuron mEPSCs were recorded for 3 min, followed a 2 min bath application of ET1/ET3 (10 nM each); a 9 min ACSF wash period and ended with 3 min of CNQX (50 µM) to block AMPA/KA receptors. **B.** Analysis of normalized data shows no significant changes in frequency, peak amplitude, decay tau or 10-90% rise time of CA3 pyramidal neuron mEPSCs during agonist application during the ACSF wash period. There was no difference in basal CA3 pyramidal neuron mEPSC frequency (insert graph in **B**).
Figure 3.5. FMRF selectively increases Ca\(^{2+}\) in CA3 astrocytes. **A.** Images of hippocampal slices from littermate control (WT) and MrgA1+ brains. Slices were bulkloaded with SR101 (left panels) and pressure loaded with OGB-1AM (middle panels). The right panel shows co-localization of the SR101 and OGB-1AM in astrocytes simultaneously imaged for both. **B.** Bath application of Gq-GPCR agonist cocktail (DHPG, histamine, carbachol; 10\(\mu\)M each, left traces) elicited robust Ca\(^{2+}\) increase in both WT and MrgA1+ astrocytes. The MrgA1+ selective agonist FMRF (15 \(\mu\)M; right traces) increased Ca\(^{2+}\) in CA3 astrocytes from MrgA1+.
hippocampal slices (bottom right trace), but failed to elicit increases in WT astrocytes (top right trace). Three representative traces are shown for IP$_3$R2 WT and IP$_3$R2 cKO for each condition. C. Table quantifying percentage of SR101 positive astrocytes that responded to application of agonist cocktail or ET1/ET3 from all experiments.
Figure 3.6. Selective Gq-GPCR-mediated Ca\textsuperscript{2+} increases in astrocytes does not affect mEPSC parameters. A. Representative traces of CA3 pyramidal neuron mEPSCs recorded from WT and MrgA1+ hippocampal slices. CA3 pyramidal neuron mEPSCs were recorded for 3 min, followed a 3 min bath application of FMRF (15 µM each); a 9 min ACSF wash period and ended with 3 min of CNQX (50 µM) to block AMPA/KA receptors. B. Analysis of normalized data shows no significant changes in frequency, peak amplitude, decay tau or 10-90% rise time of CA3 pyramidal neuron mEPSCs during agonist application or the ACSF wash period.
Chapter IV: Astrocyte IP$_3$R2-Mediated Ca$^{2+}$ Increases and Behavior

4.1. INTRODUCTION

In recent years, our understanding of the role of astrocytes in physiology has increased dramatically, with published reports supporting a role for astrocytes in control of vascular responses, modulation of neuronal activity and pathological responses (Haydon and Carmignoto, 2006; Halassa et al., 2007a; Iadecola and Nedergaard, 2007). The primary mechanism by which astrocytes are thought to modulate neuronal activity is through the release of neuroactive substances (termed gliotransmitters) such as glutamate, ATP/adenosine, and D-serine (Hamilton and Attwell, 2010). The release of gliotransmitters has been theorized to be triggered by the activation of Gq-GPCR receptors on astrocytes, which elicit IP$_3$R-dependent Ca$^{2+}$ increases from endoplasmic reticulum stores (Scemes, 2000; Fiacco and McCarthy, 2006). Immunohistochemistry has shown that IP$_3$R2 is the predominant IP$_3$ receptor subtype expressed in astrocytes, whereas neurons express IP$_3$R1 and to a lesser degree IP$_3$R3 (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007).

While there are numerous studies investigating the role of Ca$^{2+}$-dependent release of gliotransmitters in cultured cells and in situ brain slices, there is little data demonstrating a role for Ca$^{2+}$-dependent gliotransmitter release in vivo and its impact on behavior. However, several studies have been published showing a
physiological role for other astrocyte proteins in behavior. A conditional knockout of the gap junction protein connexin 43 (Cx43) in astrocytes causes enhanced locomotor activity in open field testing (Frisch et al., 2003; Theis et al., 2003). A knock out mouse model of the glial protein S100β, a Ca$^{2+}$ binding protein, leads to enhanced LTP in situ and enhanced performance in the Morris water maze, as well as changes to T-maze exploratory activity, in behavioral studies (Roder et al., 1996; Nishiyama et al., 2002). Findings from these studies indicate that alterations in astrocyte function can lead to functionally significant behavioral changes.

We previously reported that in an IP$_3$R2 full knockout mouse model, astrocytes are not capable of responding to Gq-GPCR stimulation with Ca$^{2+}$ responses from internal endoplasmic reticulum stores (Chapter II, Petravicz et al., 2008). We reported similar results in an astrocyte specific conditional knockout mouse model of IP$_3$R2 (Chapter III). To investigate the potential role of astrocyte IP$_3$R2-mediated Ca$^{2+}$ increases in vivo, we conducted behavioral testing on two different background strains of the IP$_3$R2 cKO mouse model. We present the novel findings that lack of IP$_3$R2-mediated Ca$^{2+}$ increases in astrocytes leads to behavioral alterations in the acoustic startle response and reversal of acquisition learning evaluated by the Morris water maze. Furthermore, we present the novel finding that the alteration in acoustic startle response is dependent on the strain of the mice suggesting that genetic background may influence astrocyte involvement in this behavioral reflex.
4.2. METHODS

*Generation of IP₃R2 cKO mice*

For behavioral testing, two different mouse strains were used to compare possible effects of strain differences on the IP₃R2 cKO. The first strain of mice used was generated as described in Chapter III. Briefly, IP₃R2floxed/flox mice on a Black Swiss background were crossed to C57BL/6 mice expressing Cre-recombinase under a fragment of the human GFAP promoter (Casper and McCarthy, 2006) to generate mice heterozygous for the floxed IP₃R2 allele and Cre-recombinase. These mice were then interbred with Black Swiss mice homozygous for the floxed IP₃R2 allele to generate mice heterozygous for Cre-recombinase and homozygous for the floxed IP₃R2 allele. Mice from these breeding were designated as IP₃R2 cKO Black Swiss.

The second background strain was generated by backcrossing mice from the original Black Swiss background to C57BL/6J mice (obtained from Jackson Laboratory) for four generations to generate mice heterozygous for the floxed IP₃R2 allele (IP₃R²⁺/floX). These mice were then backcrossed to GFAP-Cre recombinase mice on a C57BL/6J background to generate IP₃R²⁺/floX mice with or without Cre-recombinase. These mice were then interbreed to generate mice homozygous for the floxed IP₃R2 allele (IP₃R2floX/floX) and either heterozygous or null for Cre-recombinase. Mice from these breeding were designated as IP₃R2 cKO BL6 for purposes of differentiating them from the original strain. Mice from both strains were genotyped by PCR analysis using genomic DNA and primers specific to Cre-
recombinase and the floxed IP$_3$R2 allele. Regardless of background, mice homozygous for IP$_3$R2 flox and heterozygous for Cre-recombinase are designated as IP$_3$R2 cKO. Mice homozygous for floxed (IP$_3$R2$^{\text{flox/flox}}$) but null for Cre-recombinase are designated as WT or Control.

In all behavioral tests, unless otherwise noted, data presented is from cohorts composed of mice from the IP$_3$R2 cKO BL6 background and littermate controls. Mice were housed five to a cage and were placed in the experiment room for 20 minutes prior to testing for acclimatization of the mice to the testing environment.

**Calcium Imaging**

Hippocampal slices were prepared as previously described except the brains were sectioned in a modified slicing buffer containing the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 5 MgCl$_2$, and 1 CaCl$_2$ and bubbled with 95% O$_2$ and 5% CO$_2$. Hippocampal slices were incubated at 35-37°C for 20 min in slicing buffer containing 1µM SR101 which preferentially loads astrocytes (Nimmerjahn et al., 2004). Hippocampal slices were then transferred for 10 min to warm (35-37°C) ACSF containing the following in mM: 130 NaCl, 10 glucose, 1.25 NaH$_2$PO$_4$, 24 NaCHO$_3$, 3.5 KCl, 2.5 MgCl$_2$, and 1.5 CaCl$_2$ and bubbled with 95% O$_2$ and 5% CO$_2$. The calcium indicator Oregon Green BAPTA-AM (OGB-1AM) was suspended in 100µl ACSF containing 20% pluronic acid (final DMSO concentration 0.04%). The ACSF contained the following in mM: 150 NaCl, 2.5 KCl and 10 HEPES with the pH adjusted to 7.3-7.5 with 1M NaOH.
Hippocampal slices were placed in a perfusion chamber with a constant flow of oxygenated normal ACSF. Pipettes (1-2MΩ resistance) filled with the OGB-1AM containing ACSF were lowered to the surface of the slices and backpressure applied. The pipette was then lowered 40 µm into the hippocampal striatum to the pyramidal cell layer of the CA3 region. OGB-1AM was injected for 2-3 minutes (based on the pipette resistance) and then lowered a further 35 µm deeper into the slice and injected for an additional 2-3 minutes. The pipette was then removed and the slice transferred to room temperature (25°C) oxygenated ACSF and allowed to recover for a minimum of 45 min prior to imaging.

Astrocyte calcium increases were recorded using a two photon imaging system (Coherent Chameleon Ultra, Coherent Inc, Santa Clara, CA). Astrocytes were identified by SR101 loading and regions of interest were drawn around the SR101 positive cell bodies. Increases in average fluorescence in regions of interest indicate increase in Ca²⁺ concentration. Fold increase over baseline was calculated for each trace and reported as ΔF/F₀.

Behavioral Testing Cohorts

Behavioral testing cohorts consisted of age matched IP₃R2 cKO Black Swiss or IP3R2 cKO BL6 mice with littermate controls. IP₃R2 Black Swiss mice were only evaluated for Rotatod, Open Field and Acoustic Startle behaviors due to retinal degeneration in the background strain.
Elevated plus-maze test for anxiety-like behaviors

Mice were given one 5-min trial on the elevated plus-maze. The elevated plus-maze apparatus consisted of two open arms and two closed arms with 40 cm high walls. The maze is elevated 50 cm from the floor and the arms are 21 cm long. Mice were placed in the center area of the maze (9.5 cm x 9.5 cm), and allowed to freely explore the maze. Measures were taken of time in, and number of entries into, the open and closed arms. Percent open arm time was calculated as 100 x (open arm time/(open arm time + closed arm time)). Percent open arm entries were calculated using the same formula, but using the measure for entries.

Open Field Activity.

Exploration in a novel environment was assessed by a one hour trial in an open field (40 cm x 40 cm x 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photobeams broken during the trial in five-minute intervals, with separate measures for horizontal activity ( ), fine movements (repeated breaking of the same set of photobeams), and vertical activity (rearing 11 movements). Time spent in the center region of the activity chamber was used as a measure of anxiety-like behavior in a novel environment.
Rotarod

Mice were assessed for balance and motor coordination on an accelerating rotarod (Ugo-Basile, Stoelting Co., Wood Dale, Il). The revolutions per minute were initially set at 3 rpm, and progressively increased to 30 rpm over the course of a 5 min trial session. Each mouse was given 5 trials in total, 3 trials on the first day and 2 trials forty-eight hours later. Each trial was separated by 45 sec between trials. Latency to fall or rotate off the top of the turning barrel was measured by the rotarod timer. If the mouse immediately fell off at the beginning of the first trial, that trial was not counted, and the mouse was given a new trial.

Acoustic Startle Response

The acoustic startle measure was based on the reflexive whole body flinch following exposure to a sudden noise. Animals were tested using the San Diego Instruments SR-Lab system using the procedure described by Paylor and Crawley (Paylor and Crawley, 1997). Briefly, mice were placed in small Plexiglas cylinder within a large sound chamber (San Diego Instruments). The cylinder is place upon a piezoelectric transducer, which allowed the vibration to be detected and quantified by computer software. The chamber includes a fan, a house light, and a loudspeaker for the acoustic stimuli consisting of bursts of white noise. Background sound levels were maintained at 70 dB.

Each mouse was given one session consisting of 42 trials following a 5 min habituation period. Seven different types of trials were presented: no-stimulus trials,
trials with the acoustic startle stimulus (40 ms, 120 dB) alone, and trials with a prepulse stimulus (20 ms, either 74, 78, 82, 86, or 90 dB) delivered 100 ms before the onset of the startle stimulus. The different trial types were presented in blocks of 7, in randomized order within each block, with an average interval of 15 sec (range: 10 to 20 sec). Measures were taken of the startle amplitude for each trial, defined as the peak response during a 65-ms sampling window that began with the onset of the startle stimulus. An overall analysis was performed for each subject’s data for levels of prepulse inhibition at each prepulse sound level (calculated as 100 – [(responses amplitude for prepulse and startle stimulus together/response amplitude for the startle stimulus alone) x 100].

Morris Water Maze

Mice were assessed for spatial learning using the Morris water maze. The water maze consisted of a large circular pool (diameter = 122 cm) partially filled with water (45 cm deep, 24-26°C) located in a room with numerous visual cues. Mice were tracked by an automated system (Noldus Ehtovision 3.0) using a camera suspended above the water maze pool. Mice were tested for their ability to find an escape platform (diameter = 12 cm) on three different components: visible platform acquisition, hidden (submerged) platform acquisition, and subsequent probe trial in the absence of the platform. Following the hidden platform probe trial, the platform was moved to a new location and the mice were trained again to find the platform and given a subsequent probe trial to measure reversal learning. In both hidden and
reversal learning, the criteria for learning was an average group latency of 15 sec or less to locate a platform across four consecutive trials per day.

In the visible platform test, each animal was given four trials per day for three days to swim to an escape platform indicated by a patterned cylinder extended above the surface of the water on the hidden platform. For each trial the mouse was placed into the pool, at one of four possible locations determined randomly and given 60 sec to find the visible platform. If the mouse found the platform, the trial ended and the mouse was allowed to remain on the platform for 10-15 secs prior to the start of the next trial. If the mouse did not find the platform, the mouse was placed on the platform for 10-15 sec and then given the next trial. Measures were taken of latency to find the platform, swimming distance, and swimming velocity by the Nodulus Ethovision tracking system.

Mice were then trained on the hidden platform test. The same testing procedure as described above was used, with each animal receiving four trials per day. At the end of the day when the average group latency reached 15 sec or less, mice were given a 1 min probe trial in the pool with the platform removed. In this probe trial, selective quadrant search was evaluated by measuring percent of time spent in each quadrant of the pool. Spatial learning was demonstrated by greater swim times in the quadrant where the platform had previously been present in comparison to other quadrants in the pool. Following 1-2 days after the hidden probe trial, mice were tested for reversal learning using the same training paradigm except that the platform was located in the diagonal opposite quadrant from its previous location. Measures were taken for latency to find the platform, swimming
distance and swimming velocity. Upon reaching the 15 sec or less criterion the platform was removed from the pool and the group was given the probe trial to evaluate reversal learning.

**Tail Suspension Test**

Mice were assessed for depressive behaviors by measuring time spent immobile using the tail suspension test. Mice were suspended by the tail with tape for 5 minutes inside of a plastic, open faced box and their activity recorded. The total duration of immobility (defined as no struggling movements) for each mouse was manually scored. Percent time immobile was calculated by dividing the duration of immobility by the total duration of the trial. Percent immobile time was averaged among cohorts and reported as average + SEM.

**Statistical analysis**

For the open field activity, rotarod performance, acoustic startle response and Morris water maze tests, data was analyzed using a repeated measure ANOVA test. For the plus maze and the tail suspension tests, data was analyzed using Student's t-test. Significant differences of $p < 0.05$ were reported.
4.3. RESULTS

*IP₃R2 cKO BL6 animals do not display anxiety-like behaviors in the elevated plus maze.*

Littermate control and IP₃R2 cKO BL6 mice were tested for changes in anxiety-like and exploratory behavior in the elevated plus maze. Mice were placed in the center of the elevated plus maze and allowed to freely explore for 5 minutes. The number of entries and the time spent in the open and closed arms of the plus maze were recorded. No significant difference was found between control and IP₃R2 cKO BL6 animals for the percent of time spent in the open arms of the plus maze (Figure 4.1A). Additionally, no significant difference was found for the percent of open arm entries between controls and IP₃R2 cKO BL6 (Fig 4.1B). These data indicate the lack of IP₃R2-mediated Ca²⁺ increases in astrocytes does not affect anxiety and exploratory behavior measured by the elevated plus maze.

*IP₃R2 cKO Black Swiss or BL6 animals do not display changes in spontaneous motor function as measured by open field activity.*

Littermate control and IP₃R2 cKO Black Swiss and BL6 mice were tested for spontaneous motor function and exploratory behavior in the open field activity test. Mice were placed into the activity box and allowed to freely explore for 60 minutes. Mice from both the IP₃R2 Black Swiss and IP₃R2 BL6 backgrounds were tested. No significant difference was found in horizontal activity between control and IP₃R2 cKO mice on either background, indicating that IP₃R2 cKO mice are neither hyper- or
hypoactive and habituate normally to the open field environment (Figure 4.2A, B). Neither IP$_3$R2 cKO Black Swiss nor IP$_3$R2 cKO BL6 mice showed a significant difference in number of rearing movements compared to their littermate controls (Figure 4.2C, only IP$_3$R2 cKO BL6 data shown). Finally, there was no significant difference in the percentage of time spent in the center of the open field between IP$_3$R2 cKO BL6 and littermate controls indicating no change in anxiety like behavior (Figure 4.2D). Percent center time was not analyzed in the IP$_3$R2 cKO Black Swiss mice due to potential complications from the degenerative blindness present in these mice. Taken together, these data indicate that there are no changes to spontaneous locomotor activity or anxiety due to lack of astrocytic IP$_3$R2-mediated Ca$^{2+}$ signaling. Additionally, this lack of effect is not background strain-dependent.

**IP$_3$R2 cKO Black Swiss or BL6 mice do not display changes in motor coordination or learning measured by rotarod performance.**

Motor coordination and learning were tested by rotarod performance. Mice were given 5 trials (3 trials on day 1, followed by 2 trials 48 hours later), with a 45 second wait period between trials. Latency to fall off or rotate around the cylinder was measured and compared between control and IP$_3$R2 cKO mice. Animals from both the IP$_3$R2 cKO Black Swiss and IP$_3$R2 cKO BL6 strains were tested. No significant difference was found between control and IP$_3$R2 cKO mice on either background in the latency to fall (Figure 4.3A, B). Further, no significant difference was found in the motor learning aspect of rotarod performance over the course of
five trials. These results indicate that lack of IP₃R2-mediated Ca²⁺ increases in astrocytes has no effect on motor coordination or learning.

**IP₃R2 cKO Black Swiss mice display a strain-dependent effect on acoustic startle response, with no changes in pre-pulse inhibition.**

The acoustic startle response test is a measure of auditory processing and gating in mice (Li and Yue, 2002). The pairing of this test with a varying decibel pre-pulse tone prior to the acoustic startle tone provides a measure of sensory motor gating. Additionally, the acoustic startle response can be used as a measure of fear conditioning and stress (Davis et al., 2003). Mice were placed in a cylinder attached to a piezoelectric transducer to record the startle reflex upon administration of a 120 dB tone. Pre-pulses of varying decibel levels (4 – 20 dB above background of 70 dB) were delivered 100 ms prior to the onset of the 120 dB tone to measure pre-pulse inhibition. IP₃R2 cKO Black Swiss mice displayed a significantly exaggerated startle reflex to the 120 dB tone compared to controls (Figure 4.4A). Delivery of pre-pulses prior to the 120 dB startle attenuated the startle reflex; however the amplitude of the response remained significantly higher than controls at all pre-pulse decibel levels (Figure 4.4A). Pre-pulse inhibition in the IP₃R2 cKO Black Swiss mice remained unaffected, indicating that there was no deficit in pre-pulse inhibition (Figure 4.4C).

Surprisingly, IP₃R2 cKO BL6 mice do not exhibit an exaggerated acoustic startle response for either the 120 dB tone itself or the delivery of a pre-pulse prior to
the onset of the 120 dB tone (Figure 4.4B). A significant interaction between acoustic startle and genotype was found ($p < 0.05$), however post-hoc analysis did not find any significant differences for the 120 dB tone or at any pre-pulse decibel level. Furthermore, IP$_3$R2 cKO BL6 mice did not display any significant difference in their pre-pulse inhibition (Figure 4.4D). Taken together, these results indicate that lack of IP$_3$R2-mediated Ca$^{2+}$ increases in astrocytes has no effect on sensory motor gating measured by pre-pulse inhibition in either background strain. In contrast, there is a strain-dependent effect of the knockout on the startle reflex measured by the acoustic startle response. This may indicate a differential effect of the lack of astrocyte IP$_3$R2-mediated Ca$^{2+}$ signaling upon auditory threshold or processing. Testing of the acoustic brain stem responses will be conducted to evaluate changes to the IP$_3$R2 Black Swiss auditory processing, using IP$_3$R2 BL6 mice as a comparison. Conversely, it may indicate that IP$_3$R2 cKO Black Swiss mice have elevated stress levels leading to exaggerated startle responses. Further testing will be required to determine if elevated stress levels are a factor in this phenotype.

**IP$_3$R2 cKO BL6 mice do not display depressive behaviors in the tail suspension test**

Depressive behavior measured by the tail suspension test was compared between control and IP$_3$R2 cKO BL6 mice. Mice were suspended by the tail from the ceiling of an open front testing chamber and their activity digitally recorded. Mice were then scored manually for time immobile, defined as no signs of struggling or movement. Percent time immobile was subsequently calculated. No significant
difference was found between IP$_3$R2 cKO BL6 and control mice in percent time immobile (Figure 4.5). These data indicates that lack of IP$_3$R2 cKO BL6 in astrocytes does not affect depressive behaviors measured by tail suspension test.

*IP$_3$R2 cKO BL6 mice do not display differences in visual, acquisition or reversal learning in the Morris water maze.*

IP$_3$R2 cKO BL6 mice were tested for changes in learning and memory using the Morris water maze. Mice were tested as described in the Methods section. Briefly, mice were given three days of training using a visually cued escape platform to determine if the mice were capable of swimming and able to visually detect cues used in the water maze. No significant difference was found between IP$_3$R2 cKO BL6 and control mice in the ability to detect and swim to the visually cued escape platform (Figure 4.6).

Acquisition training of the location of a hidden escape platform was then conducted. The visual cue for the escape platform was removed and the platform moved to the opposite quadrant (from quadrant 3 to quadrant 1). Additionally, the visual cues surrounding the water maze pool were changed to provide different spatial cues for acquisition learning. Mice were then given 4 trials per day of acquisition training for the location of the hidden escape platform. The average latency to find the hidden escape platform was calculated for the behavioral cohort. Mice were considered to have met the threshold for acquisition when the cohort (defined as control and IP$_3$R2 cKO BL6 mice combined) latency reached 15 sec or
less. No significant differences were found between IP$_3$R2 cKO BL6 and control mice in their ability to acquire the location of the hidden escape platform (Figure 4.7A. Combined genotype threshold was met on day 6 for Group 1, and day 5 for Group 2).

Reversal learning involves the moving of the hidden escape platform to the opposite quadrant of the water maze pool (from quadrant 1 to quadrant 3). Mice were again given 4 trials per day of training for the relocated hidden escape platform. The average latency to find the relocated hidden escape platform was calculated for the behavioral cohort. Mice were considered to have met the threshold for reversal learning when the cohort latency reached 15 sec or less. No significant differences were found between IP$_3$R2 cKO BL6 and control mice in reversal learning of the location of the moved hidden platform (Figure 4.7B. Combined genotype threshold was met on day 6 for Group 1, and day 5 for Group 2).

**IP$_3$R2 cKO BL6 mice display a target quadrant preference acquisition, but not reversal of acquisition in the Morris water maze.**

Upon reaching the group threshold in the acquisition and reversal learning trials, the mice were given a one minute probe trial with platform removed to assess target quadrant preference. Measures were taken of the percentage of time spent in each quadrant. No significant differences were found between IP$_3$R2 cKO BL6 and controls in the probe trial performed after acquisition learning (platform located in
quadrant 1; Figure 4.8A). Within genotype repeated measures ANOVA showed significant preference for the quadrant where the hidden platform was located for both IP₃R2 cKO BL6 and control, indicating a preference for the quadrant where the platform had been located (Figure 4.8A). The probe trial performed after reversal of acquisition learning revealed a significant difference between IP₃R2 cKO BL6 and controls (Figure 4.8B). The within genotype repeated measures ANOVA revealed that while control mice displayed a significant preference for the new target quadrant (quadrant 3), IP₃R2 cKO BL6 mice showed no preference for any quadrant (Figure 4.8B). Taken together, these data indicate that IP₃R2 cKO BL6 mice do not have a deficit in acquisition of the location of the hidden platform, shown by their significant preference for the target quadrant in the acquisition probe trial. However, IP₃R2 cKO BL6 mice display a deficit in reversal of acquisition learning compared to control mice, shown by their lack of significant preference for the new target quadrant. This suggests that while their learning and memory is intact, the IP₃R2 cKO BL6 mice may have a deficit in cognitive flexibility.

Ca²⁺ Imaging of IP₃R2 cKO mice

To provide functional evidence that IP₃R2 cKO BL6 mice have reduced or abolished astrocyte Gq-GPCR-mediated Ca²⁺ increases, we performed Ca²⁺ imaging on hippocampal slices taken from sample mice from behavior experiments. Hippocampal slices were bulk loaded with SR101 and pressure loaded with OGB-1AM calcium indicator dye as described in the previous chapter (see Chapter III
Methods). Bath application of a Gq-GPCR agonist cocktail (10µM DHPG, histamine and carbachol) evoked robust Ca\(^{2+}\) increases in hippocampal astrocytes from littermate control mice (Figure 4.9; 71.4% of 28 cells, 5 slices from 3 animals). In contrast, bath application of the Gq-GPCR cocktail failed to elicit Ca\(^{2+}\) increases in hippocampal astrocytes from IP\(_3\)R2 cKO mice (Figure 4.9C; 8.6% of 23 cells, 4 slices from 3 animals). This data confirms the Ca\(^{2+}\) imaging data from Chapter II (Figure 2.2) and Chapter III (Figure 3.4) and shows IP\(_3\)R2 cKO BL6 mice have a functional lack of astrocytic IP\(_3\)R-mediated Ca\(^{2+}\) signaling in the hippocampus.
4.4. DISCUSSION

In the last 30 years, our understanding of astrocytes has progressed from the belief that astrocytes solely provide structural and metabolic support to the understanding that astrocytes play an active role in key physiological processes in the brain. Astrocytes control cerebral vasculature (Iadecola and Nedergaard, 2007), participate in synaptogenesis (Ullian et al., 2004), and are critical to the regulation of neuronal function (Halassa et al., 2007a; Verkhratsky and Kirchhoff, 2007; Olsen and Sontheimer, 2008). Astrocytes respond to neuronal activity through activation of Gq-GPCRs that stimulate IP₃R2-mediated release of Ca²⁺ from internal stores both *in situ* and *in vivo* (Fiacco and McCarthy, 2006; Agulhon et al., 2008). However, our understanding of the role of astrocytic IP₃R2-mediated Ca²⁺ release in synaptic processes governing behavioral responses is very limited.

IP₃Rs are the primary Ca²⁺ release mechanism from internal stores in most cell types (Foskett et al., 2007). Deregulation of IP₃Rs can lead to cell death and may be involved in pathological processes such as Alzheimer’s disease and epilepsy (Kuchibhotla et al., 2009; Seifert et al., 2009). There are knockout mouse models for each IP₃R subtype, with varying physiological effects. The IP₃R1 KO mouse model is embryonic lethal; the few mice that survive to birth have a life expectancy of 3-4 weeks and display seizures and ataxia (Matsumoto et al., 1996). In contrast, a double knockout of IP₃R2 and IP₃R3 does not exhibit a striking neurological phenotype similar to the IP₃R1 KO mice but displays metabolic problems and deficits in olfaction (Futatsugi et al., 2005; Fukuda et al., 2008). Further, a recent study selectively knocking down IP₃R1, IP₃R2 and IP₃R3 using
antisense oligonucleotides reported that all three knockdowns displayed antidepressant effects in the forced swim test, but no changes to rotarod or locomotor activity were found (Galeotti et al., 2008).

In this chapter we present novel findings on the role of astrocytic IP₃R2-mediated Ca²⁺ release in behavior. We report that the majority of behavioral testing showed no changes between control and IP₃R2 cKO animals. No significant differences were found in the percent open arm time or percent open entries in the elevated plus maze, indicating that lack of IP₃R2-mediated Ca²⁺ increases does not affect anxiety behaviors measured by this test. This is supported by the finding that there was no significant difference between control and IP₃R2 cKO in the percent center time measured by the open field activity task, another measure of anxiety. Spontaneous locomotor activity, as measured in the open field test, was also unaffected in IP₃R2 cKO mice indicating that the mice are not hyper- or hypoactive and habituate normally. These data contrast to other astrocyte specific genetic mouse models, such as the Cx43 knockout which displays enhanced locomotor activity and the NOS2 mutant that has changes in elevated plus maze activity (Frisch et al., 2003). These mouse models indicate that alterations in astrocyte function can lead to changes in these behavioral paradigms. Our findings suggest that IP₃R2-mediated Ca²⁺ release in astrocytes are not playing a role in modulating these particular behaviors.

Motor performance and learning were also unaffected in the IP₃R2 cKO mouse model as measured by accelerating rotarod testing. The brain area most involved in rotarod motor performance is the cerebellum. Cerebellar glial cells,
particularly Bergmann glia, respond to neuronal activity in vivo with Ca^{2+} increases (Nimmerjahn et al., 2009). However, the majority of Bergmann glial Ca^{2+} transients are due to activation of Ca^{2+} permeable AMPARs, not IP_{3}Rs (Bellamy, 2006). Further, it is unclear what role astrocytic IP_{3}R-mediated Ca^{2+} release plays in the modulation of neuronal activity in the cerebellum. The lack of a deficit in rotarod performance in IP_{3}R2 cKO mice may indicate that IP_{3}R2 is not a key player in the Ca^{2+}-dependent regulation of motor performance by cerebellar astrocytes. Calcium imaging experiments will be required to determine if Bergmann glia display any changes in Ca^{2+} signaling in IP_{3}R2 cKO mice.

No significant difference was found in the percent immobility time in the tail suspension test in IP_{3}R2 cKO mice. This is somewhat surprising since another measure of depression, the forced swim test, was found to be significantly different in mice that were treated with an antisense phosphodiester nucleotide (dODNs) against the IP_{3}R2 protein (Galeotti et al., 2008). Further testing of the IP_{3}R2 cKO mice in the forced swim test may reveal test specific alterations in depressive behaviors not revealed by the tail suspension test. It is interesting to note however that while doses of phosphodiester nucleotides (ODNs) were effective at reducing IP_{3}R1 expression, the authors did not find any side effects known to occur in the IP_{3}R1KO mouse model. In contrast, equal concentration of ODNs for IP_{3}R2 or IP_{3}R3 caused convulsions and death in mice; problems associated with IP_{3}R1 deletion which may indicate some nonspecific effects of the dODNs (Matsumoto et al., 1996; Galeotti et al., 2008).
IP₃R2 cKO mice displayed a strain-dependent difference in their reaction to acoustic startle. IP₃R2 cKO mice on the original Black Swiss background strain displayed an exaggerated startle response to the 120 dB tone, even when the tone was preceded by a pre-pulse, whereas mice from the C57BL/6 backcrossed strain were unaffected. Surprisingly, while the Black Swiss background strain displayed an exaggerated response at all pre-pulse decibel levels, the pre-pulse inhibition was not significantly different than controls. This may indicate IP₃R2-mediated Ca²⁺ release in astrocytes or GFAP-expressing cell are involved in modulating auditory processing, potentially a modification in auditory threshold, but not in sensory motor gating in certain mouse strains. Another potential explanation is that acoustic startle can be affected by stress upon the subject (Davis et al., 2003). Alternatively, the exaggerated startle response of IP₃R2 cKO mice on the original Black Swiss background strain may reflect a chronic elevated level of stress that is not present in the IP₃R2 cKO C57BL/6 backcrossed mice. Further testing involving acoustic brain stem responses or stress responses may provide valuable insights into understanding this phenotype.

Perhaps the most surprising finding of these experiments is that IP₃R2 cKO BL6 mice only display significant deficits in specific aspects of spatial learning measured by the Morris water maze. The current literature concerning the role of IP₃R-mediated Ca²⁺-dependent release of gliotransmitters argues that astrocytes are critical for the formation of LTP and heterosynaptic depression (Fellin and Carmignoto, 2004; Halassa et al., 2009a; Hamilton and Attwell, 2010). Numerous reports show that blockade of Ca²⁺ increases in astrocytes leads to impairments in
or blockage of LTP (Yang et al., 2003; Serrano et al., 2006; Henneberger et al., 2010), which has been argued to be the neuronal basis of learning and memory in the Morris water maze (Cole et al., 1998). IP₃R2 cKO BL6 mice showed no deficit over repeated trials in learning the location of a visually cued platform, a hidden escape platform during acquisition training or a relocated escape platform during reversal training. Furthermore, IP₃R2 cKO BL6 mice showed no significant difference in quadrant selectivity in a single trial probe test following acquisition training compared to controls. However, IP₃R2 cKO BL6 mice show a deficit in quadrant selectivity following reversal of acquisition learning. This may indicate that IP₃R2 cKO BL6 mice have less cognitive flexibility or impaired synaptic plasticity. It should be noted that while many studies use Morris water maze as a measure of learning and memory, it does not directly relate to electrophysiological LTP (Cain, 1997; D'Hoooge and De Deyn, 2001). Further, Morris water maze does not solely involve the hippocampus but also the striatum, basal forebrain, cerebral cortex and cerebellum. Further anatomical and electrophysiological studies involving plasticity in those brain regions may yield insight into this phenotype.

Learning and memory in the Morris water maze may be related to changes in protein synthesis and the formation of new neuronal connections (Thomas et al., 1994; Meiri and Rosenblum, 1998). Astrocytes are known to be a major source of brain-derived neurotrophic factor (BDNF) in the adult brain (Friedman et al., 1998). BDNF is involved in synapse maturation in the CNS and is critical for activity-dependent plasticity (Cohen-Cory et al., 2010). Cultured astrocytes are known to produce BDNF in response to Gq-GPCR activated, IP₃R-mediated Ca²⁺ increases,
potentially providing an activity dependent pathway to increase in BDNF (Jean et al., 2008). Thus, blockade of IP₃R-mediated Ca²⁺ increases in astrocytes may lead to reduced levels of BDNF in response to neuronal activity and may affect either the formation or stability of new synaptic contacts in the brain, leading to a deficit in reversal acquisition in the Morris water maze.

Overall, we have shown that lack of IP₃R2-mediated Ca²⁺ increases in astrocytes leads to distinct behavioral changes in the Morris water maze and a strain-dependent change in acoustic startle response. Elevated plus maze, rotarod performance and open field activity were unaffected in this animal model. These results provide a significant advancement in our understanding of the role of IP₃R-mediated Ca²⁺ increases in astrocytes and their role in physiological behaviors in vivo. Further studies using different behavioral paradigms such as Barnes maze, a less stressful behavioral test for learning and memory, are currently being conducted to further characterize the IP₃R2 cKO mouse model to elucidate unknown roles of astrocytes in regulating behavior.

In conclusion, it is extremely striking that given the multiple proposed roles of astrocyte Ca²⁺-dependent release in the modulation of synaptic transmission that the IP₃R2 cKO mice are relatively normal. A large number of high profile papers and reviews have been published concerning the critical role of astrocytes in vascular tone (Zonta et al., 2003; Mulligan and MacVicar, 2004; Takano et al., 2006), neuronal migration (Platel et al., 2010), synaptogenesis (Nagler et al., 2001; Christopherson et al., 2005), and synaptic transmission (Fellin et al., 2004; Serrano et al., 2006; Jourdain et al., 2007; Perea and Araque, 2007; Gordon et al., 2009).
Our findings in this chapter are reflective of the argument that the majority of evidence for IP$_3$R-mediated, Ca$^{2+}$ dependent release of gliotransmitters is either the result of pharmacological or non-physiological stimulation and does not reflect the physiological role of astrocytes in the CNS.
4.5. Figures and Tables

Figure 4.1. Loss of IP₃R2-mediated Ca²⁺ increases in astrocytes does not affect anxiety measured by the elevated plus maze. Littermate control and IP₃R2 cKO BL6 mice were evaluated for changes in anxiety-like behavior using the elevated plus maze test. 

A. There were no significant differences between littermate control and IP₃R2 cKO BL6 mice for percent time spent in open arms (Control, n = 26; IP₃R2 cKO, n = 25; p > 0.05).

B. There were no significant differences between littermate control and IP₃R2 cKO BL6 mice for percent entries into open arms (Control, n = 26; IP₃R2 cKO, n = 25; p > 0.05). Data are presented as mean ± SEM.
**Figure 4.2.** Blockade of IP$_3$R2-mediated Ca$^{2+}$ increases does not affect spontaneous motor function or anxiety measured by open field activity. Mice were tested in an open field activity box to monitor changes in spontaneous activity. **A, B.** No significant differences were found in horizontal activity between littermate control and IP$_3$R2 cKO animals from either background strain (**A.** IP$_3$R2 Black Swiss: Control, n = 22; IP$_3$R2 cKO, n = 24; p > 0.05 and **B.** IP$_3$R2 BL6: Control, n = 26; IP$_3$R2 cKO, n = 26; p > 0.05). **C.** No significant difference were found in rearing movements in IP$_3$R2 cKO BL6 mice (Control, n = 26; IP$_3$R2 cKO, n = 26; p > 0.05). **D.** Percent center time was not significantly different between IP$_3$R2 cKO BL6 and littermate controls (Control, n = 26; IP$_3$R2 cKO, n = 26; p > 0.05). Data are presented as mean ± SEM.
Figure 4.3. Lack of IP$_3$R2-mediated Ca$^{2+}$ increases does not affect rotarod performance. No significant difference was found in the rotarod performance between littermate controls and IP$_3$R2 cKO mice on either background. A. IP$_3$R2 cKO Black Swiss mice (Control, n = 22; IP$_3$R2 cKO, n = 24; p > 0.05). B. IP$_3$R2 cKO BL6 mice (Control, n = 26; IP$_3$R2 cKO, n = 26; p > 0.05). X-axis indicates trial number. Data are presented as mean ± SEM.
Figure 4.4. Lack of IP₃R2-mediated Ca²⁺ increases has a strain-dependent effect on the acoustic startle response. Both strains of mice were tested for sensory motor gating in the acoustic startle response test. **A.** IP₃R2 cKO Black Swiss mice display an exaggerated startle response during testing compared to littermate controls (Control, n = 14; IP₃R2 cKO, n = 18; main genotype effect: p < 0.01). **B.** IP₃R2 cKO BL6 mice displayed no significant difference compared to littermate controls in the acoustic startle response test (Control, n = 25; IP₃R2 cKO, n = 26; p > 0.05). **C.** No significant difference was found for pre-pulse inhibition in the IP₃R2 cKO Black Swiss background strain (Control, n = 14; IP₃R2 cKO, n = 18; p > 0.05) or **D.** IP₃R2 cKO BL6: Control, n = 25; IP₃R2 cKO, n = 26; p > 0.05). Data are presented as mean ± SEM. ** p < 0.01; * p < 0.05.
**Figure 4.5.** Blockade of IP$_3$R2-mediated Ca$^{2+}$ increases does not affect depressive behaviors measured by tail suspension test. No significant difference was found in the percent immobile time upon tail suspension between control and IP$_3$R2 cKO BL6 mice (Control, n = 15; IP$_3$R2 cKO, n = 14; p > 0.05).
Figure 4.6. Lack of IP₃R2-mediated Ca²⁺ increases does not affect the latency to find a cued visible platform in the Morris water maze. No significant differences were found between littermate control and IP₃R2 cKO BL6 mice on the visible platform training. Control, n = 26; IP₃R2 cKO, n = 26; p > 0.05. Data is presented as mean (± SEM) of four trials per day.
Figure 4.7. Loss of IP$_3$R2-mediated Ca$^{2+}$ increases does not affect acquisition or reversal of acquisition learning in the Morris water maze. **A.** No significant difference was found between littermate control and IP$_3$R2 cKO BL6 acquisition learning of the location of a hidden platform (Left panel: Group 1, p > 0.05; Right panel: Group 2, p > 0.05). **B.** No significant difference was found between littermate control and IP$_3$R2 cKO BL6 mice in the reversal of acquisition learning of the relocated hidden platform (repeated measures ANOVA: Left panel: Group 1, p > 0.05; Right panel: Group 2, p > 0.05). In both acquisition and reversal of acquisition, experimental groups differed in the length of time to reach threshold therefore the data was not combined. (Group 1: Control, n = 16; IP$_3$R2 cKO, n = 16 and Group 2: Control, n = 10; IP$_3$R2 cKO, n = 9).
Figure 4.8. IP$_3$R2 cKO BL6 mice display selective target quadrant preference during acquisition but not reversal of acquisition in the Morris water maze. Mice were given a one minute probe trial with the hidden platform removed. The target quadrant is indicated by the black bar. **A.** Both littermate controls and IP$_3$R2 cKO BL6 mice spent significantly more time in the target quadrant following acquisition (Control: n = 26, p < 0.001; IP$_3$R2 cKO: n = 26, p < 0.001). **B.** Littermate control mice exhibit a significant preference for the target quadrant while IP$_3$R2 cKO BL6 mice do not display a preference (Control: n = 25, p < 0.01; IP$_3$R2 cKO: n = 25, p > 0.05). Data is presented as mean ± SEM. p-values represent within-group ANOVA for significant main effect of target quadrant.
**Figure 4.9.** IP₃R2 cKO BL6 astrocytes do not display Gq-GPCR, IP₃R-mediated Ca²⁺ increases. **A.** Images of hippocampal slices from littermate control (IP₃R2 WT) and IP₃R2 cKO BL6 brains. Slices were bulkloaded with SR101 (left panels) and pressure loaded with OGB-1AM (middle panels). The right panel shows co-localization of the SR101 and OGB-1AM in astrocytes simultaneously imaged for both. **B.** Bath application of a Gq-GPCR agonist cocktail (DHPG, histamine, carbachol; 10µM each) failed to elicit increases in astrocyte Ca²⁺ in IP₃R2 cKO astrocytes (right traces). The agonist cocktail elicited robust Ca²⁺ increases in IP₃R2 WT astrocytes. Three representative traces are shown for IP₃R2 WT and IP₃R2 cKO BL6. **C.** Table quantifying percentage of SR101 positive astrocytes that responded to application of agonist cocktail from all experiments.
Chapter V: General Discussion and Future Directions

5.1. General Discussion

The field of neuroscience has undergone a significant shift in the understanding of synaptic function in the last several decades. Communication between pre- and postsynaptic elements of the synapse through transmitter release was thought to only involve neurons. The first indication that astrocytes were involved in synaptic communication occurred when cultured astrocytes were found to respond to the neurotransmitter glutamate with intracellular Ca\(^{2+}\) increases (Cornell-Bell et al., 1990; Porter and McCarthy, 1997). Subsequently, astrocytes were reported to respond to a wide variety of neurotransmitters with increases in intracellular Ca\(^{2+}\) following agonist application or neuronal stimulation (Agulhon et al., 2008). A functional role for astrocytic Ca\(^{2+}\) increases was reported by Parpura et al. (1994), who found that increases in astrocyte intracellular Ca\(^{2+}\) following stimulation with a Gq-GPCR agonist led to glutamate release and neuronal NMDAR-mediated Ca\(^{2+}\) elevations. This placed astrocytes in a position to modulate synaptic transmission in an activity-dependant manner and forever changed how the synapse was thought to function. These findings provided the basis for the theory of gliotransmission and the tripartite synapse, with astrocytes playing an integral role in synaptic communication alongside neuronal elements (Araque et al., 1999).
The study of gliotransmission has rapidly become a prominent area of research in the neuroscience, with evidence concerning this theory published in many high profile journals. Gliotransmission, regardless of the model system used, is currently defined as the astrocyte-derived release of transmitters dependent upon activation of astrocytic Gq-GPCRs and subsequent Ca\(^2+\) from internal stores (Haydon and Carmignoto, 2006). Published data providing evidence for gliotransmission and modulation of synaptic activity include changes in excitatory and inhibitory synaptic transmission (Kang et al., 1998; Angulo et al., 2004; Fellin et al., 2004; Liu et al., 2004b; Parri and Crunelli, 2007; Perea and Araque, 2007; Gordon et al., 2009; Ortinski et al., 2010), heterosynaptic depression and long term potentiation (Yang et al., 2003; Pascual et al., 2005; Serrano et al., 2006; Andersson et al., 2007a; Henneberger et al., 2010). Gliotransmission has recently been expanded to include regulation of physiological processes such as cerebral vascular responses, respiration, and homeostatic sleep pressure (Halassa et al., 2009b; Koehler et al., 2009; Gourine et al., 2010).

5.2. Evidence for Gliotransmission Is Not Monolithic

Despite what appears to be an overwhelming amount of data implicating astrocytes and gliotransmission in regulating synaptic transmission, a critical view of the literature reveals some potential issues. First, there are discrepancies in the evidence for gliotransmission between cultured astrocytes and astrocytes \textit{in situ}. While it has been firmly established that cultured astrocytes release glutamate, ATP
and D-serine, the currently defined gliotransmitters, the mechanisms by which release occurs differ among reports. Release of gliotransmitters from cultured astrocytes can occur by vesicular release (Kreft et al., 2009), connexin hemichannels (Stout et al., 2002; Ye et al., 2003), volume regulated anion channels (Takano et al., 2005; Kimelberg et al., 2006; Ramos-Mandujano et al., 2007) and large conductance P2X7 receptors (Duan et al., 2003; Fellin et al., 2006a; Suadicani et al., 2006). In contrast, astrocytes in situ are argued to release gliotransmitters primarily through vesicular release (Halassa et al., 2009a; Hamilton and Attwell, 2010). Second, the source of Ca\textsuperscript{2+} to trigger vesicular release from astrocytes has not been clearly defined in culture. There are data implicating voltage gated calcium channels, IP\textsubscript{3} receptors, ryanodine receptors, store operated calcium channels and mitochondria in the regulation of Ca\textsuperscript{2+}-dependent exocytosis of gliotransmitters from astrocytes (Volterra and Steinhauser, 2004; Montana et al., 2006; Reyes and Parpura, 2009). However, astrocyte release of gliotransmitters in situ is generally believed to occur through Gq-GPCR activated, IP\textsubscript{3}R receptor mediated Ca\textsuperscript{2+} increases (Fiacco and McCarthy, 2006; Scemes and Giaume, 2006). Finally, in situ studies of the same physiological processes have yielded different, though not always conflicting, results. Heterosynaptic depression of Schaffer collateral-CA1 synapses in the hippocampus in acute hippocampal slices was reported to be modulated by Ca\textsuperscript{2+}-dependent vesicular release of both ATP/adenosine and glutamate from astrocytes (Pascual et al., 2005; Serrano et al., 2006; Andersson et al., 2007a). Similarly, the formation and dynamic range of long term potentiation at
this synapse is affected not only by ATP/adenosine release from astrocytes, but D-serine as well (Henneberger et al., 2010).

Research on the effects of gliotransmission has a critical limitation: the methods researchers have used to stimulate or block intracellular Ca\(^{2+}\) increases in astrocytes are either non-selective or induce nonphysiological responses in astrocytes (Agulhon et al., 2008; Fiacco et al., 2009). Application of endogenous ligand or specific agonists to astrocyte Gq-GPCRs suffer from the limitation that the same Gq-GPCRs are nearly all also expressed by neurons. Mechanical stimulation and intense depolarization likely result in damage to the astrocyte, thereby eliciting a nonphysiological and potentially pathological Ca\(^{2+}\) response. Lastly, uncaging of either Ca\(^{2+}\) or IP\(_3\) in astrocytes bypasses much of the second messenger systems regulating vesicular release and produces an intracellular Ca\(^{2+}\) response of nonphysiological spatial and temporal dynamics. Conversely, widespread use of the calcium chelator BAPTA provides support for the Ca\(^{2+}\) dependency of gliotransmission, but also affects all Ca\(^{2+}\)-dependent process in astrocytes.

Tools to selectively increase or block Gq-GPCR-activated, IP\(_3\)R-mediated intracellular Ca\(^{2+}\) increases were necessary. Two mouse models were developed to address this need: the MrgA1 mouse model and IP\(_3\)R2 KO mouse model, which selectively activates or blocks Gq-GPCR, IP\(_3\)R-mediated intracellular Ca\(^{2+}\) increases in astrocytes, respectively. The MrgA1 mouse model has revealed that selective activation of astrocytic Gq-GPCR signaling does not lead to the release of glutamate to affect excitatory synaptic transmission in the hippocampus (Fiacco et al., 2007). Further, activation of astrocyte intracellular Ca\(^{2+}\) increases does not affect the
formation or maintenance of long term potentiation (Agulhon et al., 2010). While these data argue that Gq-GPCR activation of astrocyte Ca\(^{2+}\) increases does not trigger gliotransmitters release, the possibility remained that only certain Gq-GPCR elicited release of gliotransmitters. Support of this possibility was found by Shigetomi et al (Shigetomi et al., 2008), who discovered that activation of PAR-1, but not P2Y1 receptors led to activation of neuronal NMDA receptors. However, this is inconsistent with the literature, where numerous Gq-GPCRs have been shown to increase Ca\(^{2+}\) and elicit gliotransmitter release from astrocytes (Table 1, Agulhon et al., 2008).

Regardless of the GPCRs involved, the field is unified in the concept that Ca\(^{2+}\)-dependent gliotransmission plays an important role in synaptic transmission and plasticity, and by extension, the function of the central nervous system. All studies using chelation of intracellular Ca\(^{2+}\) by BAPTA demonstrate that intracellular Ca\(^{2+}\) is involved in mediating the astrocyte derived effects on synaptic activity. It was published that astrocytic intracellular Ca\(^{2+}\) increases were necessary and sufficient to trigger the release of gliotransmitters in response to neuronal activity (Parpura and Haydon, 2000). Therefore, the general consensus in glial biology was that Ca\(^{2+}\) increases in astrocytes were absolutely critical to gliotransmitter release. This viewpoint held that genetic deletion of IP\(_3\)R-mediated Ca\(^{2+}\) increases in astrocytes would lead to severe neurological problems, vascular issues and potential lethality.

Evidence in this thesis demonstrates that IP\(_3\)R2 is the sole functional IP\(_3\)R expressed by astrocytes in situ (Petravic et al., 2008). This is in full agreement with
immunocytochemical data as well as functional data from cultured astrocytes (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007; Weerth et al., 2007). Further, genetic deletion of IP₃R2 in astrocytes completely blocks Gq-GPCR activated, IP₃R-mediated Ca²⁺ release from intracellular stores in all brain regions examined (Petravicz et al., 2008) Chapter II and III]. Contrary to the view of the field, IP₃R2 KO mice display no readily detectable physiological phenotype and on a gross level appear completely normal.

Astrocytic intracellular Ca²⁺ increases, whether agonist-evoked or spontaneous, are argued to trigger the release of gliotransmitters to modulate synaptic activity (Zhang and Haydon, 2005; Halassa et al., 2009a; Parpura and Zorec, 2010). Surprisingly, no evidence of deregulation or impairment of synaptic activity was found in electrophysiological recordings in hippocampal neurons of IP₃R2 cKO mice. Synaptic activity measured by spontaneous and miniature EPSCs, was unaffected in mice with deletions of IP₃R2 (Petravicz et al., 2008). IP₃R2 KO mice showed no change in the frequency of sEPSCs in CA1 pyramidal neurons, contradicting predictions that in the absence of gliotransmission, there would be significant changes in sEPSC frequency (Nadkarni and Jung, 2007). Further, CA3 pyramidal neuron excitatory synaptic transmission was also found to be unaffected in response to genetic removal of IP₃R-mediated Ca²⁺ increases in the IP₃R2 cKO model, or selective activation of Gq-GPCR, IP₃R-mediated Ca²⁺ increases in the MrgA1 model.

Astrocytes are argued to be involved in the regulation of physiological processes governing behavior (Halassa and Haydon, 2010). Studies involving the
blockade of IP$_3$R-mediated Ca$^{2+}$ signaling in astrocytes and its effect on behavior has been lacking until now. Our data show that relatively few of the behaviors examined were affected by lack of IP$_3$R2-mediated Ca$^{2+}$ increases in vivo. IP$_3$R2 cKO mice displayed an exaggerated acoustic startle response; however this effect was found to be strain-dependent, indicating the possible influence of factors unrelated to the lack of astrocyte Ca$^{2+}$ increases. This strain-dependent release may also reflect elevated stress levels or changes to auditory processes by peripheral factors in the affected mouse strain. Further testing such as measurements of auditory threshold may elucidate the mechanism governing this strain-dependent difference.

5.3. Does Gliotransmission Occur?

The data presented in this thesis, in addition to Fiacco et al (2007) and Agulhon et al (2010), at first glance appear to directly contradict the current state of the literature and the theory of gliotransmission. This perception is incorrect, as the data presented herein merely suggest that the mechanism by which gliotransmission is thought to occur needs to be reexamined, specifically in regards to the necessity of IP$_3$R-mediated intracellular Ca$^{2+}$ increases in astrocytes. This is supported by the current evidence for gliotransmission that has used either non-selective or non-physiological stimuli to elicit Ca$^{2+}$ increases in astrocytes. These methods may produce experimental artifacts which can be interpreted as positive data for gliotransmission. For example, non-physiological Ca$^{2+}$ increases induced by
uncaging of IP$_3$ do not travel through the cell with the same spatial profile as endogenous GPCR activation but can trigger the release of gliotransmitters (Fiacco and McCarthy, 2004). Further, neuronal synaptic activity shows no modulation upon selective activation of astrocyte intracellular Ca$^{2+}$ increases in the MrgA1 mouse model. However, experiments using uncaging of IP$_3$ in MrgA1 astrocytes displayed increases in the frequency of AMPA receptor-mediated sEPSCs, demonstrating that gliotransmission can occur from astrocytes in this model in a stimulus-dependent manner (Fiacco et al., 2007).

We propose that if gliotransmission does occur under physiological conditions, IP$_3$R-mediated Ca$^{2+}$ increases are not the predominant mechanism by which Ca$^{2+}$ triggers release of gliotransmitters. Recently, spontaneous near membrane Ca$^{2+}$ events, not associated with Gq-GPCR activation, were observed using a membrane tethered genetic Ca$^{2+}$ indicator protein in cultured astrocytes (Shigetomi et al., 2010). These authors argue that based on their findings, that large global Ca$^{2+}$ increases triggered by bath application of Gq-GPCR agonists are fundamentally different than spontaneous and neuronal activity evoked increases and may therefore represent non-physiological responses. This is inconsistent with the fact that nearly all reports of gliotransmission involve global Ca$^{2+}$ increases in astrocytes upon either uncaging of Ca$^{2+}$ or IP$_3$, or activation of Gq-GPCRs. Our findings contradict the concept that Gq-GPCR, IP$_3$R-mediated Ca$^{2+}$ increases in astrocytes cause gliotransmission. Activation of the MrgA1 receptor causes global Ca$^{2+}$ increases in astrocytes similar to pharmacological activation of commonly used Gq-GPCR agonists without effecting synaptic transmission (Fiacco et al., 2007;
Agulhon et al., 2010). Blockade of global intracellular Ca\textsuperscript{2+} increases in the IP\textsubscript{3}R2 KO mouse model has no affect on basal synaptic transmission or long term potentiation (Petravicz et al., 2008; Agulhon et al., 2010). These findings represent a significant conflict in the theory of gliotransmission that remains unresolved.

While the focus of Ca\textsuperscript{2+} signaling in astrocytes has been on IP\textsubscript{3}R-mediated increases, there are alternative mechanisms. Calcium permeable AMPA receptors represent a mechanism for Ca\textsuperscript{2+} entry into glial cells. These receptors are expressed on several glial cell types including NG2 cells (Ge et al., 2006; Hamilton et al., 2009), oligodendrocytes (Butt, 2006), and Bergmann glia (Bellamy, 2006). Astrocytes in the hippocampus of juvenile mice are known to express functional Ca\textsuperscript{2+}-permeable AMPA receptors (Porter and McCarthy, 1995b; Seifert and Steinhauser, 1995). AMPA receptors on astrocytes during development show an increase in expression of receptor subunits of relatively low Ca\textsuperscript{2+}-permeability but still maintain appreciable levels of Ca\textsuperscript{2+} permeable behavior (Seifert et al., 2003). Further evidence for AMPA receptor expression on astrocytes was obtained via functional imaging using the sodium sensitive dye SFBI-AM in hippocampal astrocytes \textit{in situ} (ages P12-P21). This study reveals significant intracellular sodium transients in astrocytes in response to neuronal afferent stimulation (Langer and Rose, 2009). Sodium signals using SFBI-AM were recorded in SR101 positive astrocytes with passive membrane properties indicative of protoplasmic hippocampal astrocytes. While the majority of the sodium signal in these cells was blocked by the glutamate transporter TBOA, roughly 20% of the signal was
attributed to AMPA receptors. It remains to be determined if the AMPA receptor component of the signal is from Ca$^{2+}$-permeable or impermeable receptors.

There exists potential for a novel mechanism by which astrocytes may increase intracellular Ca$^{2+}$ levels. Electrogenic uptake of both GABA and glutamate from the synaptic cleft has been extensively demonstrated in astrocytes (Bergles and Jahr, 1997, , 1998; Diamond and Jahr, 2000). Intracellular sodium increases as a result of the electrogenic requirements for transporter uptake of GABA or glutamate have been demonstrated by functional imaging studies in astrocytes of juvenile and young adult mice (Doengi et al., 2009; Langer and Rose, 2009). Doengi et al. (2009) proposed that GABA transport by astrocytes caused elevations in intracellular sodium levels in olfactory bulb astrocytes, thereby reducing the activity of the sodium/calcium exchanger. This led to an increase in intracellular Ca$^{2+}$ sufficient to activate Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from IP$_3$ receptors. While this mechanism was shown only in juvenile animals, Langer and Rose (2009) used both juvenile and young adult mice (P12-P22) when showing significant increases in glutamate transporter mediated sodium concentrations in hippocampal astrocytes. This raises the possibility of this release mechanism occurring in mature astrocytes, which express sodium/calcium exchangers (Blaustein et al., 2002; Minelli et al., 2007). Evidence for a sodium/calcium exchanger modulation of Ca$^{2+}$ signals exists in cultured astrocytes in response to potassium depolarization (Paluzzi et al., 2007), osmotic swelling (Rojas et al., 2008), AMPA receptor activation (Smith et al., 2000) and in response to glutamate transporter activity (Rojas et al., 2007). Finally, astrocytes in the visual cortex of ferrets display finely tuned Ca$^{2+}$ increases that
mapped in tight correlation to neuronal responses induced by orientation and spatial frequency stimuli (Schummers et al., 2008). These activity-dependent Ca\(^{2+}\) increases in astrocyte were blocked by the glutamate transporter antagonist TBOA, implicating glutamate transport as a necessary component to the Ca\(^{2+}\) increases. These findings fit with the concept that transporter generated Ca\(^{2+}\) signals involving sodium and the sodium/calcium exchanger could underlie a physiological mechanism for intracellular Ca\(^{2+}\) increases both independent of and involving IP\(_3\)R-mediated Ca\(^{2+}\) release. Further, it would be possible to generate small, localized microdomains of Ca\(^{2+}\) signals via a mechanism depending on the level of transporter activity. Langer and Rose (2009) demonstrated activity-dependent scaling of transporter mediated sodium increases in patch-loaded hippocampal astrocytes that could potentially lead to localized Ca\(^{2+}\) increases through a glutamate transporter-sodium/calcium exchanger mechanism.

While both of the mechanisms mentioned above present alternative Ca\(^{2+}\) sources that could be involved in regulated exocytosis, there exists no data in astrocytes *in situ* or *in vivo* to support this. However, it is evident that studies into alternative mechanisms of intracellular Ca\(^{2+}\) increases in astrocytes will be crucial to understanding the role of astrocyte Ca\(^{2+}\) signaling in physiology and pathology.

**5.4. Future Directions**

Overall, our findings suggest that astrocytic Gq-GPCRs are not directly coupled to the regulation of synaptic transmission. What then is the role(s) of
astrocytic Gq-GPCR signaling in physiology? While unclear, there are several possibilities that should be considered. For example, Ca\(^{2+}\) increases in astrocytes through activation of Gq-GPCRs leads to the activation of phospholipase A\(_2\) (PLA\(_2\)) to trigger arachidonic acid (AA) mobilization (Koehler et al., 2009). Astrocytic AA is then used as a substrate for several enzymatic pathways that produce vasoreactive compounds such as PGE\(_2\) and 20-HETE (Gordon et al., 2007) Therefore, the blockade of IP\(_3\)R-mediated Ca\(^{2+}\) increases in the IP\(_3\)R2 cKO mouse model may lead to deficits in vascular changes regulated by astrocytes in response in neuronal activity. *In vivo* studies examining vascular responses in the IP\(_3\)R2 cKO mice will be important in evaluating this possibility.

The regulation of gene transcription in astrocytes by IP\(_3\)R-mediated Ca\(^{2+}\) increases presents another avenue of further research. Astrocytes produce BDNF in response to Gq-GPCR activation of the PLC/IP\(_3\) pathway (Jean et al., 2008), a secreted factor involved in synapse formation and maintenance. Further, data exists from cultured astrocytes that intracellular Ca\(^{2+}\) is involved in the pathways governing the expression of matrix metalloproteinase (MPP)-9 (Wu et al., 2009), cyclooxygenase-2 (COX-2) and Rcan 1-4 (an calcineurin inhibitor) (Canellada et al., 2008), as well as c-fos transcription (Edling et al., 2007) which are involved in several physiological processes. This raises the distinct possibility that IP\(_3\)R2 cKO astrocytes will have altered transcriptomes. Gene array studies like Cahoy et al. (2008) could provide valuable insight into genes regulated by Ca\(^{2+}\) increases in astrocytes and direct future areas of research.
Finally, astrocytic Ca\textsuperscript{2+} signaling has been shown to be altered in AD (Kuchibhotla et al., 2009), cerebral ischemia (Ding et al., 2009), and thought to contribute to pathological states such as epilepsy (Seifert et al., 2010). The IP\textsubscript{3}R2 cKO mouse model represents an ideal in vivo model system to study the role and deregulation of Ca\textsuperscript{2+} signaling pathways in these pathological processes. Research using these mice could provide potential drug targets against astrocyte Ca\textsuperscript{2+}-dependent pathways to develop treatments against neurological diseases.

In conclusion, the role of IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} signaling in astrocytes has been presented as a critical component of their role in nervous system function, particularly in respect to synaptic function. The theory of gliotransmission has become an increasingly accepted, though still controversial, concept in neuroscience, resulting in publication of numerous review articles in high profile journals and textbooks. The data presented in this thesis, supported by Fiacco et al. (2007) and Agulhon et al. (2010), present a direct challenge to the current theory of gliotransmission, which gliobiologists and the neuroscience community needs to acknowledge and take into consideration.
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