UNIVERSITY OF CALIFORNIA
RIVERSIDE

Gq G Protein Coupled Receptor Mediated Potentiation of Astrocytic Glutamate Uptake

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

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August 2011

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ACKNOWLEDGEMENTS

A single experiment involving electrophysiology and calcium imaging requires the use of tools, instruments and equipments ranging from nylon stockings to laser scanning confocal microscope. In addition to all the predecessors who established this status quo of science and technology for conducting sophisticated experiments, a few who partook in the endeavor culminating in this dissertation study deserve my special thanks and gratitude.

The University of California, Riverside, despite being a young University provided the basic amenities sufficient for undertaking this dissertation work and I am indebted to the institution. At UCR, much of the credit for instilling the critical thinking abilities and the hard-working nature in me goes to my first mentor Dr. Glenn I. Hatton. He was a great man with a wise mind, a tender heart and a sarcastic tongue. I owe some of the technical skills to Dr. Yu-Feng Wang, who was a post-doc in the Hatton Lab. Fortune had its tide and I floated with it to Dr. Fiacco's fledgling laboratory.

Kudos to Dr. Fiacco for his great support and being available to discuss even the smallest questions and ideas I had. Words defy me in describing our symbiotic relationship. In Dr. Fiacco's lab, an excess of supportiveness dwindled the constraints of resources. My lab-mates during the three years in Dr. Fiacco's lab – Min-Yu, Alison, Tom and Kelli are unique and I could learn at least some thing from each one of them. I cherish the multitude of experiences in Dr. Fiacco's team.

I am thankful to the members of my dissertation committee - Dr. Currie gave me adequate freedom and Dr. Razak was a good friend and guide. With Dr. Razak, I also had the privilege to talk neuroscience in my native language Tamil. The very wise and critical piece of advice and support always came from the graduate advisor, Dr. Adams – a man I revere for his panoramic view of biology. Perla, the graduate secretary, helped in sorting out the red tape despite my occasional acrid attitude.

I attribute to the direct and indirect influences of my Dad, Mr. V. Devaraju, the shaping of my interest in science, especially neuroscience. The thrust these influences gave me was strong enough to overcome the odd circumstances in Hatton Lab which forced me to switch labs. I would like to thank my younger brother Jaga and sister Jai for their encouragement and my elder brother Ramesh for his discouragement for the ‘6 year long Ph.D.’ My Mom, Mrs. Leelavathi has been an over-the-phone companion during all my lonely days in the deserted Riverside and I thank her for the unfathomable love.

I would like to thank the people of India and the United States of America for their tax dollars which supported me financially during the period before and at UCR. My thanks also go to a few devoted teachers at Madras Veterinary College and all those who supported my formal education which included fourteen years in college.

Dedicated to Nature

ABSTRACT OF THE DISSERTATION

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University of California, Riverside, August 2011
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Astrocytic glutamate transporters, GLAST and GLT-1 (rodent analogs of EAAT1 and EAAT2, respectively) constitute the major pathway of glutamate uptake in the central nervous system. Uptake of synaptically released glutamate by astrocytes is essential for maintaining a healthy level of excitatory activity in the brain. It is also known that astrocytes *in situ* possess a myriad of G-protein coupled receptors (GPCRs) of the G_i, G_s, and G_q families. Stimulation of glutamatergic afferents not only leads to astrocytic glutamate uptake but also has been shown to stimulate group I (Gq type) metabotropic glutamate receptor (mGluR) mediated intracellular Ca^{2+} elevations in astrocytes both *in vivo* and *in situ.* Activation of mGluRs has been associated with short and long-term plastic changes of glutamate transport in cultured astroglia and in Purkinje neurons in cerebellar slices. This led us to hypothesize that astrocytic Gq GPCRs modulate glutamate transport. This hypothesis was tested in acute mouse hippocampal

slices using two different approaches: (1) activation of astrocytic mGluRs in slices from wild-type mice using a tetanic high frequency stimulus (200Hz, 1s) applied to the Schaffer collaterals (SCs); (2) activation of a transgenic Gq GPCR (the MrgA1R) which is selectively expressed by astrocytes. Synaptically evoked glutamate transporter currents (STCs) were isolated from the total evoked astrocytic currents and analyzed for changes in amplitude, rise time, rise slope and decay time constant. High frequency stimulation (HFS) of SCs led to potentiation of the amplitude of the STCs without changes in kinetics. Similar potentiation was not observed in the presence of group I mGluRs or the PKC inhibitor, PKC 19-36, suggesting that HFS induced potentiation of glutamate uptake is group I mGluR - PKC dependent. Activation of the MrgA1Rs selectively expressed by astrocytes also potentiated the STC amplitude indicating the sufficiency of astrocytic Gq GPCR activation for potentiation of glutamate uptake. Surprisingly, the amplitude of the slow inward astrocytic current due to potassium uptake is also enhanced following activation of either the endogenous mGluRs or the astrocyte-specific MrgA1Rs. These findings collectively suggest that astrocytic Gq GPCR activation has a synergistic modulatory effect on the uptake of both glutamate and potassium.

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Chapter 1

Introduction

1.1. A Brief Historical Backdrop

The term “neuroglia” was coined by the famous German pathophysiologist Rudolf Virchow in 1856. It is noteworthy to quote Virchow’s words here – ‘Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system in its real relations in the body, it is extremely important to have a knowledge of that substance also which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree’. More than 150 years have passed since Virchow’s lecture. The science of studying the nervous system has only recently in the past couple of decades started giving due diligence to the non-neuronal elements in the nervous system. One of the very important, though not surprising reason for physiologists being enthralled with neuronal cell types is the measurability of electrical activity rendered easier by technical advances in electronics. It took another set of technical advances, this time in optical imaging to bring the glial cells to the forefront. With the advent of fluorescence microscopy, it was discovered that astrocytes, hitherto considered as silent connective tissue, responded to neuronal activity and neuro ligands with an increase in intracellular calcium. Suddenly, the neurophysiologist who often

pulls off his electrode and tosses it when encountering the dull, electrically passive cell is taken aback upon finding a new treasure trove of the nervous system – The Glial Cells (Kettenmann and Verkhratsky, 2008).

After more than a century of silence in the biology of glial cells, small whispers started to appear. Glenn Hatton, one of the proponents for the field of Glial-Neuronal interactions and my previous mentor recounted to me that he, like most neuroscientists, was originally a neurocentric until Charles Tweedle (his collaborator) showed him the electron micrographs in which astrocytic processes seemed to have retracted in the supraoptic nuclei of lactating rats. As more and more people like Glenn Hatton gave up their neurocentric position and started studying glia, the field of glial biology blossomed with the first edition of a separate journal titled ‘Glia’ in 1988. More than a decade later, there was a boisterous outcry with the finding that astrocytes in culture exhibit calcium dependent glutamate release (Parpura et al., 1994). Such was the nature of this outcry that the noise it created sidelined the more physiologically relevant homeostatic functions of astrocytes. Due to the advances in cell culture techniques and also because it is easier to culture astrocytes compared to neurons, culture based studies took preponderance despite their physiological irrelevance. A publication in 1996 showed that astrocytes in hippocampal slices respond to stimulation of Schaffer collaterals with increases in calcium (Porter and McCarthy, 1996). It was followed by another in 1997 also based on hippocampal slice model showing that when astrocytes take up glutamate inward

currents are produced (Bergles and Jahr, 1997). Such findings of greater physiological relevance had to wait another decade for further exploration until the noise created by cultured astrocytes exhibiting calcium dependent glutamate release was filtered out in 2007 by the rebuttal from a slice based study (Fiacco et al., 2007). It is at this chronological juncture, this dissertation work on the modulation of astrocytic glutamate transport by astrocytic Gq GPCRs is put forth.

1.2. Heterogeneity of Astrocytes

It is widely agreed that the non-neuronal cell types in the brain, called glial cells, fall into three major categories – Astrocytes, Microglia and Oligodendrocytes. The name ‘astrocyte’ was coined by Michael von Lenhossek in 1893. Slightly later, Kolliker and Andriezen divided astrocytes into fibrous and protoplasmic subtypes (reviewed in Kettenmann and Verkhratsky, 2008). Fibrous astrocytes are located in the white matter and have long processes that run between myelinated fibers, some of which extend into gray matter. By contrast, protoplasmic astrocytes are ramified, have many short processes, and are located in the gray matter (reviewed in Wolfgang Walz, 2000). More specialized astrocyte-related cells are also present in the adult mammalian CNS: Ependymal cells – form a continuous lining around the ventricles and the central canal; Tanycytes – Specialized ependymal cells which have long processes extending to the pial wall; Pituicytes – astrocyte like cells located in the posterior pituitary; Bergmann glia – found only in cerebellum; Muller cells – specialized glial cells

found in the retina; Radial glia – found mainly in the developing brain (reviewed in Wolfgang Walz, 2000). Of late, it has been recognized that there might be subtypes within the protoplasmic astrocytes. There are certain cells similar to astrocytes in morphology and found mainly in juvenile animals (P4-P21 rat). These cells express a connective tissue marker, NG2 and have a relatively higher input resistance (~200 mΩ) and exhibit voltage-gated outward and inward potassium currents. In the mouse hippocampal slices, we encounter these types of cells mostly up to P12-P13 and are carefully excluded from experiments by looking at their electrophysiological profile (Lin and Bergles, 2002; Zhou et al., 2006). This study involves the protoplasmic astrocytes in the stratum radiatum of hippocampal CA1 region. Only those protoplasmic astrocytes exhibiting a passive electrophysiological profile are included in this study and are referred to hereafter for the most part as just 'astrocytes'.

All the experiments described in this study were conducted in mouse hippocampal slices. Though mouse is a very useful experimental mammal because of its size and the highly developed mouse genetics, differences have been found between rodent and human astrocytes. The human counterparts are 2.6-fold larger in diameter and extend 10-fold more GFAP (glial fibrillary acidic protein)-positive primary processes (Oberheim et al., 2009). The human neocortex also harbors several anatomically defined subclasses of astrocytes not represented in rodents. These include a population of astrocytes that reside in layers 5-6 of the neocortex and extend long fibers characterized by regularly

spaced varicosities. Another specialized type of astrocyte, the interlaminar astrocyte, abundantly populates the superficial cortical layers and extends long processes without varicosities to cortical layers 3 and 4 (Oberheim et al., 2009).

1.3. Physiological Roles of Astrocytes

A single protoplasmic astrocyte extends thousands of fine membranous processes that surround synapses and blood vessels (Ventura and Harris, 1999; Bushong et al., 2002). A longstanding notion that astrocytes are mere space fillers was followed by a sudden increase in astrocyte research when methods for preparing purified astrocyte cultures were developed (McCarthy and de Vellis, 1980). A plethora of studies demonstrated that astrocytes in culture expressed a wide variety of metabotropic receptors for neuro-ligands (Porter and McCarthy, 1997). The technical limitation of studying astrocytes in intact preparations was overcome by the first time demonstration that metabotropic glutamate receptors on astrocytes respond to neuronally released glutamate in hippocampal slices (Porter and McCarthy, 1996). The initial studies on cultured astrocytes - besides helping to draw attention to these cells - also introduced some high impact findings such as calcium dependent vesicle mediated glutamate release (Parpura et al., 1994) and propagation of calcium waves across a network of astrocytes (Cornell-Bell et al., 1990; Dani et al., 1992). Both of these phenomena were later proven to be an artifact observable only in cultured astrocytes as calcium dependent glutamate release was not observed in hippocampal slices (Fiacco et

al., 2007) and calcium waves were not observed in slices (Fiacco et al., 2007) or *in vivo* (Schummers et al., 2008). Recently intercellular calcium waves within a network of astrocytes was observed in live mice with amyloid-β plaques but not in wild-type controls suggesting the pathological nature of intercellular calcium waves (Kuchibhotla et al., 2009).

Current research on astrocytes suggests the following major physiological roles for astrocytes: 1) Glutamate uptake (Bergles et al., 1999; Danbolt, 2001; Tzingounis and Wadiche, 2007) 2) Metabolic coupling (Magistretti, 2006) 3) Potassium buffering (Kofuji and Newman, 2004) 4) Water homeostasis (Simard and Nedergaard, 2004) 5) Hemodynamic regulation (Iadecola and Nedergaard, 2007). Though I have separated these five different processes for the purpose of discussion, these are highly inter-related. For example, glutamate uptake is most often accompanied by potassium uptake (Bergles and Jahr, 1997) and is tied to metabolism as tricarboxylic (TCA) acid cycle is activated upon glutamate uptake (Magistretti, 2006). Activation of aerobic TCA cycle increases oxygen consumption leading to increased blood circulation (Gordon et al., 2008). Uptake of potassium and glutamate introduce changes in osmolarity requiring water homeostasis (Simard and Nedergaard, 2004). As this study is mainly concerned with glutamate uptake, I will briefly review some of the physiological roles of astrocytes other than glutamate uptake followed by an elaborate discussion of glutamate uptake and its regulation.

1.3.1. Metabolic Coupling

Astrocytes are suggested to have a key role in coupling neuronal activity to glucose utilization. Sodium-coupled re-uptake of glutamate by astrocytes and the ensuing activation of the Na-K-ATPase triggers glucose uptake and its glycolytic processing, resulting in the release of lactate from astrocytes. Lactate can then contribute to the activity-dependent fuelling of the neuronal energy demands associated with synaptic transmission. A large body of experimental evidence led to the proposal of a model for metabolic coupling called the 'astrocyte–neuron lactate shuttle' (Magistretti, 2006; Rouach et al., 2008).

1.3.2. Potassium Buffering

Depolarization of glial cells following nerve impulses was first demonstrated in frog glial cells (Orkand et al., 1966) and a potassium spatial buffering hypothesis was proposed. This hypothesis states that astrocytes take up excess extracellular potassium ions, distribute them via their gap junction-coupled cell syncytium, and extrude the ions at sites in which extracellular potassium concentration is low. A weakly rectifying inward rectifying potassium channel Kir4.1 was demonstrated to be predominantly expressed in glia (Takumi et al., 1995). Kir4.1 was found to be localized in astrocytic processes surrounding synapses and blood vessels and in oligodendrocyte cell bodies (Holthoff and Witte, 2000; Poopalasundaram et al., 2000; Higashi et al., 2001). K^+ spatial buffering has been best investigated extensively in the retina, where it is called

K^+ siphoning to reflect the directed K^+ transport from the plexiform layers to the vitreous and to blood vessels (Kofuji and Newman, 2004). A recent study on conditional knock out of astrocytic Kir4.1 reported reduced spontaneous neuronal activity of hippocampal CA1 neurons and greatly elevated post-tetanic potentiation and short-term potentiation suggesting a role for Kir4.1 astrocytic potassium uptake in modulating synaptic strength. When we measure astrocytic currents following Schaffer collateral stimulation, there is a significant contribution of potassium current in addition to the glutamate transporter current and is subtracted out to isolate the transporter component (described in detail in Chapter 2).

1.3.3. Water Homeostasis

The water channel Aquaporin4 (AQP4) is the main aquaporin in the brain and is predominantly expressed by astrocytes. The channel is present along the entire plasma membrane, including the processes that ensheath glutamatergic synapses, but is concentrated in those astrocyte membrane domains that form end feet around the cerebrovasculature. Evidences demonstrating colocalization of AQP4 with Kir4.1 in the retinal Muller cells and coupling of potassium and water flux in cortical slices suggest that astrocytes aid in water movement and homeostasis in the brain (reviewed in Amiry-Moghaddam and Ottersen, 2003).

1.3.4. Hemodynamic Regulation

A rise in intracellular free calcium concentration ($[Ca^{2+}]_i$) within astrocyte endfeet, which collectively circumscribe all cerebral vessels, can initiate vasoconstriction or vasodilation (Filosa et al., 2004; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Takano et al., 2006). A recent finding has delineated the role of astrocyte derived lactate in hemodynamic regulation (Gordon et al., 2008). In high oxygen, extracellular Prostaglandin E_2 (PGE_2) is rapidly cleared by prostaglandin transporters as a result of low extracellular lactate levels. This keeps extracellular PGE_2 levels low and allows astrocyte-derived arachidonic acid to constrict arterioles. During more active periods, the drop in partial pressure of oxygen and the rise in extracellular lactate and adenosine promote astrocyte-mediated dilation. High external lactate hinders PGE_2 clearance and increases extracellular PGE_2, which dilates arterioles.

1.4. Glutamate Uptake

1.4.1. Types of Glutamate Uptake Systems

The following paragraph is an excerpt from an excellent review by Niels C. Danbolt in Progress in Neurobiology (Danbolt, 2001). The total amount of glutamate, the major excitatory amino acid neurotransmitter in the mammalian nervous system, is about 10 mmol/kg wet weight. Of this total amount of glutamate only about 1-3 μM is found in the extracellular space. Thus the difference in concentration across the plasma membrane is several thousand-

fold. Glutamate, unlike some other transmitters like acetylcholine, is not metabolized in the synaptic cleft. There are specialized uptake systems with low and high affinity that maintain the concentration gradient across plasma membranes. The high affinity uptake of glutamate is sodium dependent and is mediated by transporter proteins located both in the plasma membrane of glial cells and neurons. The K_m value of this high affinity uptake system ranges from 1-20 µM due to variation in the methodology across studies. The low affinity uptake system is not well characterized and its existence as a separate entity is controversial. This uptake system has been reported to have K_m values above 500 µM. Other sodium independent glutamate uptake systems such as chloride dependent high affinity uptake in mitochondria and cystine-glutamate exchanger in a variety of cell types have also been reported. Uptake systems for dicarboxylic amino acids are widely distributed in the body in the liver, small intestine, fibroblasts and kidney. Inside the cell, glutamate transporters exist on mitochondrial membranes and in the synaptic vesicles of glutamatergic neurons. Vesicular glutamate transporters (VGLUTs) accumulate glutamate by a mechanism very different from those on plasma membrane. Vesicular glutamate uptake is independent of sodium and potassium and has a lower affinity (K_m about 1 mM). VGLUTs use the proton gradient generated by vacuolar H^+-ATPase to transport glutamate inside the vesicle.

1.4.2. Types of Plasma Membrane Sodium Dependent Glutamate Transporters and their Localization

The following description of the classification and localization of glutamate transporters is excerpted from two reviews (Danbolt, 2001; Robinson, 2006). Sodium-dependent high affinity glutamate uptake in mammals is mediated by a family of five proteins that share 50% sequence similarity. Originally the non-human homologs of three of these transporters were termed GLAST (glutamate aspartate transporter), GLT-1 (glutamate transporter subtype 1), and EAAC1 (excitatory amino acid carrier 1); the human homologs were named EAAT1 (for excitatory amino acid transporter 1), EAAT2, and EAAT3, respectively. In addition, there are two other members of the family, EAAT4 and EAAT5. There are also variants of some of these transporters that arise by alternate messenger RNA (mRNA) splicing (Robinson, 2006). The five transporters are differentially distributed throughout the nervous system. Expression of GLAST/EAAT1 is found on glial cells throughout the nervous system, but is particularly enriched in apparently specialized glia, such as the Bergmann glia in the cerebellum, supporting glia in the vestibular end organ and Muller cells of the retina. GLT-1/EAAT2 is also found on astrocytes with some variation in expression throughout the brain and somewhat lower levels in the spinal cord. Neither GLT-1 nor GLAST appears to be uniformly distributed on the membrane, suggesting there are mechanisms that restrict distribution to specific domains on the plasma membrane. There are variants of GLT-1 that arise from alternate mRNA splicing, and at least one of these variants is found on presynaptic nerve terminals.

EAAC1/EAAT3 is found on neurons and some white matter cells, particularly developing oligodendroglia. EAAC1 expression is enriched in neurons that are primarily associated with glutamatergic signaling. It is found on perisynaptic regions of dendrites and on cell bodies. EAAC1 is also found on γ-amino butyric acidergic (GABAergic) neurons. In contrast to the other glutamate transporters, EAAC1 immunoreactivity is found in the cytoplasm of cells throughout the nervous system, implying that there is a pool of transporters available for trafficking to the plasma membrane. EAAT4 is enriched in Purkinje cells of the cerebellum where expression is highest on the cell body and spines, but there are also low levels of EAAT4 in some forebrain neurons. EAAT5 expression is restricted to retina.

A sodium dependent medium affinity glutamate uptake system has been reported in the neuromuscular junction of arthropods (Danbolt, 2001). There are astrocyte-like cells in simpler organisms like Drosophila. The neuromuscular junction in drosophila is glutamatergic and is surrounded by glial cells exhibiting properties similar to mammalian astrocytes such as glutamate uptake, synaptically induced calcium transients, coupling to tracheal structures mediating gas exchange similar to end feet in mammals (Danjo et al., 2011). Despite the heterogeneity of protoplasmic astrocytes among the three eukaryotic species discussed here - drosophila, mouse and humans - it is interesting to note that the glutamate uptake function of astrocytes remains conserved, confirming the notion that astrocytes serve as the third component of the synapse formed between

presynaptic and postsynaptic neuronal components. Prokaryotes also express a variety of glutamate uptake mechanisms for concentrative uptake of metabolites. Five different glutamate uptake systems are identified in *E. coli*. Corynebacterium glutamicum secretes large amounts of the amino acid and is used for industrial production. In these simpler organisms, glutamate might be used as a metabolite (Slotboom et al., 1999; Danbolt, 2001).

1.4.3. Glutamate Transporter Structure and Mechanism of Transport

Transport of glutamate has been found to be coupled to the co-transport of 3 Na^+, one proton, and $glutamate^{(-)}$ and the countertransport of 1 K^+ (Zerangue and Kavanaugh, 1996). In addition to the transport of these ions, a thermodynamically uncoupled bidirectional chloride ion conductance also occurs during glutamate transport (Wadiche et al., 1995). This chloride transport is thought to be mediated by a channel-like region on the N-terminal side of the protomers (Yernool et al., 2004). Presence of an alternate access mechanism for glutamate transport and a channel-like mechanism for chloride transport within the same molecule is intriguing and is actively researched for biophysical understanding of these mechanisms (Kavanaugh, 1998). It is noteworthy to mention here that although the currents mediated by the major astrocytic transporters GLAST and GLT-1 are usually identified as a single transporter current, there exists some difference in the associated chloride transport between the transporters. At positive potentials, GLAST transfected oocytes

exhibited significant outward current due to chloride transport whereas GLT-1 transfection did not elicit any outward current up to +60 mV, suggesting that GLAST, but not GLT-1, mediated transport has a significant chloride component (Wadiche et al., 1995). There is also a difference in transporting some glutamate analogs. (2S, 4R)-4-methylglutamate is a substrate for GLAST (Km = 54 µM) but a competitive blocker of GLT-1 (Ki = 3.4 µM). Though the experiments in this study did not differentiate between the two types of transporters, it will be very informative to test such differential nuances between the two transporter types. While the biophysical studies reached a conclusion on the stoichiometry of the ions co- and counter-transported with glutamate, the binding order is still unresolved and depends on the conditions of the study. If the protons bind before glutamate, it would suggest that the protons released from vesicles might be priming the transporters for uptake (recall that vesicular glutamate transporters fill vesicles with both protons and glutamate) (Tzingounis and Wadiche, 2007). Another important point to be observed here is that glutamate transporters are transporters of not only glutamate but also protons and hence might be involved in acid-base regulation.

The structural information described in this paragraph is an excerpt from a review classifying transporters based on structure-function relationships (Slotboom et al., 1999). Transporters which use free energy stored in ion and/or solute gradients over the membrane to drive transport are classified as secondary transporters. Glutamate transporters form a unique family of

secondary transporters with three different groups: C4-dicarboxylate transporters (found in bacteria), glutamate/aspartate transporters (found in bacteria and eukaryotes) and neutral amino-acid transporters (found in bacteria and eukaryotes). The glutamate/aspartate transporters group includes the sodium dependent high affinity glutamate transporters described earlier. Sixty protein sequences from Eucarya, Bacteria, and Archaea, belonging to the glutamate transporter family were found in the BLAST facility. The sequences vary in length from 396 to 581 residues, with the bacterial ones being significantly shorter (396 to 491 residues) than the eukaryotic ones (479 to 581 residues). A stretch of about 150 residues in the C-terminal part of the sequences is better conserved than the N-terminal part. The primary amino acid sequence of prokaryotic and eukaryotic transporters has diverged considerably. The hydropathy profile on the other hand is better conserved reflecting conservation of the global structure. Based on hydropathy, site-directed mutagenesis and glycosylation state, the N-terminal half has been found to contain 6 transmembrane alpha helices and the C-terminal half to have 4 transmembrane regions (7-10), two of them being alpha helices. A conserved intracellular motif which is a serine- and threonine-rich stretch in the hydrophilic region membrane spanning-helix 6 is an important locus for phosphorylation and regulation by protein kinases.

The analytical methods used to derive the structural analyses described in the earlier paragraph did not use crystallography. The crystal structure of the bacterial transporter from *Pyrococcus horikoshi* was determined in 2004 (Yernool

et al., 2004) and agrees well with earlier reports which relied mainly on hydropathy and mutagenesis analysis. The quaternary structure of the transporter is assembled as a bowl shaped trimer. Each protomer consists of 8 transmembrane regions and two hair-pin loops (1-6 transmembrane alpha helices on the N-terminal half; two transmembrane alpha helices – 7 &10 and two hair-pin loops – 8 & 9, on the C-terminal half). The hairpin loops are predicted to serve as the extracellular and intracellular gate of the transporter. The substrate binding site is located roughly half way deep inside the membrane. A motif which was suggested to be involved in substrate binding has been found to be located in helix 7 on the C-terminal half (Vandenberg et al., 1995). The quaternary structure also reports the substrate binding site on the C-terminal half. The translocation pore is predicted to be located in helix 10 as it has amphipathic residues (Conradt and Stoffel, 1995; Slotboom et al., 1996). The predicted mechanism of glutamate transport envisions a conformational change in the transporter upon substrate binding which will flip-flap the hairpin loops (8 and 9). Sequential opening and closing of the hairpin loops might expose the substrate binding site towards the extracellular and intracellular sides leading to translocation of glutamate and the associated ions (Yernool et al., 2004). Bacterial homologs of the sodium dependent glutamate transporters, unlike eukaryotic transporters, are not dependent on counter-transport of potassium for reverting to the unbound confirmation (Slotboom et al., 1999). In the eukaryotic astrocytic glutamate transporter, GLT-1, a glutamate residue (E404) and a

tyrosine residue (Y403) have been identified to be necessary for the potassium coupling of the transport (Kavanaugh et al., 1997; Zhang et al., 1998). Once the hairpin loops flip flap to expose glutamate, sodium and the proton to the intracellular side, these molecules might leave the transporter. A potassium ion binding to E404 and Y403 might then provide the energy needed for restoring the original confirmation of the transporter as it is transported down the concentration gradient to the extracellular side.

1.4.4. Glutamate Transporters Influence Specificity of Excitatory Synapses

The average peak concentration of glutamate in the synaptic cleft has been estimated to be 1.1 mM with a decay time constant of 1 ms. This concentration will saturate synaptic NMDA receptors and will only reach an occupancy of about 60% at AMPA receptors (Clements et al., 1992; Clements, 1996). The concentration inside the cleft is brought down rapidly by glutamate uptake and diffusion. Glutamate uptake by astrocytes is a relatively slower process compared to the glutamate transient inside the cleft. The 20-80% rise time of transporter currents in astrocytes is about 2-3 ms with a decay τ of ~20 ms at room temperature. The presence of high densities of transporters near the synaptic cleft compensates for the slower kinetics, and allows for rapid reduction in glutamate concentrations through the act of binding to the high affinity transporters alone. This also means that location and expression levels of transporters are more important in shaping the glutamate transient inside the

cleft rather than kinetics of the transporters. Synapses which are tightly wrapped by astrocytes will have a high degree of specificity compared to synapses which do not have an astrocytic process nearby, thereby permitting transmitter spill-over to adjacent synapses (Bergles et al., 1999; Huang and Bergles, 2004). It has been estimated that only about 50% of the synapses in the CA1 region of hippocampus have an apposed astrocytic process and nearly 2/3rd of the glutamate released at a synapse in this region can diffuse to neighboring synapses (Ventura and Harris, 1999). It is not surprising that in this structural arrangement glutamate uptake has been found to modulate the spatio-temporal interactions of glutamate transients from neighboring synapses (Arnth-Jensen et al., 2002).

1.4.5. Glutamate-Glutamine Cycle

In addition to the predominant expression of the glutamate transporters, astrocytes are also uniquely endowed with certain enzymes which help in the metabolism of glutamate. Astrocytes have the highest expression of glutamine synthetase, the enzyme which transaminates glutamate into glutamine (Norenberg and Martinez-Hernandez, 1979). Expression of glutamine synthetase is also reported in oligodendrocytes but not neurons (D'Amelio et al., 1990). The simplest version of the glutamate-glutamine cycle suggests conversion of glutamate to glutamine by astrocytes and then the released glutamine is returned

to neurons via glutamine transporters. Once taken up by neurons, glutamine can be resynthesized into glutamate (Danbolt, 2001).

The following excerpt from a review on glutamate uptake (Danbolt, 2001) provides a detailed overview of the glutamate-glutamine cycle. Though the glutamate-glutamine cycle is conceived as a one-step recycling of glutamate, it is more complicated than this simple notion. After uptake into astrocytes, glutamate may be metabolized through two different pathways: (a) It may be amidated to glutamine by the ATP-dependent, glutamine synthetase or (b) it may be converted to α-ketoglutarate (through deamination by glutamate dehydrogenase or by transamination by one of the transaminases). α-ketoglutarate may be metabolized through the tricarboxylic acid (TCA) cycle to succinate, fumarate and malate, successively. Malate may be metabolized further through the TCA cycle, or it may be decarboxylated to pyruvate and reduced to lactate. Both glutamine and lactate are exported from astrocytes to the extracellular fluid from which they may enter neurons. The export of glutamine from astrocytes to the extracellular fluid appears to be mediated through system N glutamine transporter SN1 and possibly also through the AST2 amino acid transporter. Neurons take up glutamine from the extracellular fluid by specific glutamine transporters - system A transporters, SAT1 and SAT2, which are abundantly expressed on glutamatergic neurons throughout the CNS. Astrocytic export and neuronal uptake of lactate occurs through different monocarboxylate transporters.

In addition to the predominant expression of glutamine synthetase, two key enzymes - pyruvate carboxylase and cytosolic malic enzyme are restricted to astrocytes. Pyruvate carboxylase is involved in de novo synthesis (from glucose) of the carbon skeleton of glutamate, and Malic enzyme is involved in returning the carbon skeleton of excess glutamate to the metabolic pathway for glucose oxidation. As a consequence of these innovations, neurons constantly require new carbon skeletons from astrocytes to sustain their TCA cycle. When these supplies are withdrawn, neurons are unable to generate amino acid transmitters and their rate of oxidative metabolism is impaired. Astrocytes might be providing, via glutamine and lactate, the carbon skeleton needed in neurons for TCA cycle and glutamate synthesis (reviewed in Hertz et al., 1999).

1.5. Gq GPCRs and Astrocytes

1.5.1. G Protein Coupled Receptors

A family of heterotrimeric guanine nucleotide binding proteins (G-proteins) plays an essential transducing role in linking cell surface receptors to effector proteins. G proteins are heterotrimers, composed of three distinct subunits: α (molecular mass = 39-46 kDa), β (37 kDa) and γ (8 kDa). The β and γ subunits exist as a tightly associated complex that functions as a unit. The alpha subunits have a single, high-affinity binding site for guanine nucleotides (GDP or GTP). The GDP-bound form of alpha binds tightly to βγ and renders the trimer inactive. The GTP-bound form of α dissociates from βγ and α and βγ subunits can then

serve as effector proteins. The identity of the α-subunit is used to identify an individual G protein oligomer. Three major named G proteins based on the type of alpha subunits are $G\alpha_s$, $G\alpha_i$ and $G\alpha_q$,referred to generally as G_s, G_i and G_q. Of these three different types of G proteins, activation of G_s and G_i leads to activation and inhibition, respectively, of adenylate cyclase. G_q activation on the other hand leads to activation of Phospholipase C (Gilman, 1987; Hepler and Gilman, 1992).

1.5.2. Gq GPCR Signaling

Receptors which are coupled to G-proteins are called G-protein coupled receptors (GPCRs) or metabotropic receptors. Activation of Gq GPCRs leads to exchange of GTP for the GDP bound to Gα subunit. The GTP bound Gα subunit then dissociates from the βγ subunit to activate phospholipase C (PLC). This activation process is thought to involve unplugging a molecular plug on the active site of the enzyme (Taylor et al., 1991; Waldo et al., 2010). Once PLC is activated, it cleaves the membrane bound phosphatidylinositol(4,5)bis-phosphate (PIP_2) into inositol(1,4,5)tris-phosphate (IP_3) and diacylglycerol (DAG). IP_3 can bind to receptors on the endoplasmic reticulum and release calcium. DAG remains bound to the membrane and activates protein kinase C (PKC). Calcium release by IP_3 and PKC activation by DAG, though considered as two separate limbs of the pathway, have a synergistic interaction (Berridge and Irvine, 1984). The action of PLC is terminated by a second domain on PLC which accelerates

hydrolysis of GTP by the α subunit to form the α-βγ complex again (Waldo et al., 2010).

1.5.3. Metabotropic Glutamate Receptors on Astrocytes

There are 8 known GPCRs for which glutamate serves as a ligand and these are named metabotropic glutamate receptor 1 (mGluR1) to mGluR8 (Lagerstrom and Schioth, 2008). Based on amino acid sequence identity, the eight mGluRs are divided into 3 groups – I, II and III. Group I includes mGluR1 and mGluR5, group II, mGluR2 and mGluR3, and group III, all others (Conn and Pin, 1997). Of the three groups of mGluRs, group I is coupled to Gq. Groups II and III are coupled to Gi (Alexander et al., 2007). Astrocytes have been shown to express group I and group II mGluRs (Porter and McCarthy, 1997). Of the two mGluRs in group I, mGluR5 was thought to be the one present on hippocampal astrocytes based on an earlier immunolocalization study (Romano et al., 1995). There is definitive evidence for presence of mGluR5 on hypothalamic astrocytes (Van Den Pol et al., 1995). A very recent publication confirms the presence of mGluR5 (of group I) and mGluR2/3 on peripheral astrocytic processes using immunogold electron microscopy (Lavialle et al., 2011). While it has been initially thought that mGluR1 (of group I) is not present on astrocytes, a recent finding suggests otherwise. *In vivo*, application of the mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) or t-ACPD evokes robust Ca^{2+} transients in cortical astrocytes, while co-application of the mGluR1 antagonist LY367385 and

the mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) reduces the whisker-evoked Ca^{2+} response in astrocytes by more than 70%. MPEP and LY367385 alone are equally effective in inhibiting the whisker-evoked response (40–50% inhibition), suggesting that both mGluR1 and mGluR5 contribute to astrocyte Ca^{2+} responses (Wang et al., 2006). The available evidence thus suggests that astrocytes express mGluRs that are coupled to activation of PLC (mGluRs 1 & 5 – group I) and also mGluRs that are coupled to adenylate cyclase (mGluR 3 – group II). Whether the group I and II mGluRs are present on the same or different population of astrocytes, or even within separate domains of the same astrocyte, is unknown. The focus of this dissertation is on the Gq subtype of Group I mGluRs on astrocytes (mGluRs 1 & 5) which are coupled to PLC.

1.5.4. Gq GPCRs on astrocytes which respond to ligands other than glutamate

Astrocytes express a surprising array of different Gq GPCRs. For example, hippocampal CA1 stratum radiatum astrocytes respond to ligands that activate purinergic, adrenergic, muscarinic, GABAergic, histaminergic, endothelin, endocannabinoid, bradykinin, thrombin, opiate, and substance P receptors with an increase in Ca^{2+} (Duffy and MacVicar, 1995; Pasti et al., 1997; Porter and McCarthy, 1997; Verkhratsky et al., 1998; Shelton and McCarthy, 1999b; Shelton and McCarthy, 2000; Serrano et al., 2006; Bowser and Khakh, 2007; Fiacco et al., 2007; Lee et al., 2007; Navarrete and Araque, 2008). A

single hippocampal astrocyte can respond to at least 4 different neuroligands (Shelton and McCarthy, 2000). It remains unclear whether different astrocytic GPCRs on a single cell regulate different physiological processes.

1.6. Acute Modulation of Glutamate Uptake by GPCRs

Transporters in general can be modulated by altering two kinetic parameters - the affinity of the transporters for the substrate (K_m) and the maximum velocity of transport (V_{max} = number of transporters x turn-over rate). Rate of turn-over is also referred to as catalytic efficiency. Turn-over rate and K_m are affected by experimental conditions and changes are difficult to interpret with certainty. A change in the number of transporters leading to changes in uptake has been described for the insulin dependent glucose transporter. A similar mechanism has been postulated for glutamate transporters and has been tested extensively in culture preparations (Robinson, 2006).

Glutamate transporters have a consensus PKC phosphorylation site. Recall that there is a highly conserved serine- and threonine-rich stretch in the hydrophilic intracellular region following helix 6, which can be phosphorylated. The presence of this consensus sequence led to the testing of PKC-dependent phosphorylation mediated regulation of glutamate uptake in HeLa cells transfected with GLT-1 and it was found that phorbol ester, a PKC activator, upregulated glutamate uptake (Casado et al., 1993b). When a serine residue within the consensus PKC dependent phosphorylation site was mutated, phorbol

ester mediated potentiation of glutamate uptake was abolished. This study for the first time demonstrated that PKC dependent phosphorylation can potentiate glutamate uptake. Following this study, a large body of work on various cultured cell types expressing different subtypes of glutamate transporters has provided a muddled picture at best for whether or not astrocyte glutamate transport is acutely modulated by GPCRs or their signaling molecules. Several groups have studied the effects of mGluR and/or PKC on GLT-1/GLAST activity and obtained contradictory results showing that activation of PKC increases, decreases, or has no effect using HEK293, C6 glioma, MCB or L-M(TK-) cells, astrocyte-neuron co-cultures, or cultured primary astrocytes (Casado et al., 1991; Hansson and Ronnback, 1991; Casado et al., 1993a; Gonzalez and Ortega, 1997; Daniels and Vickroy, 1999; Tan et al., 1999; Kalandadze et al., 2002; Aronica et al., 2003; Vermeiren et al., 2005). Other groups have reported that the glutamate transporters themselves autoregulate their trafficking to the membrane (Duan et al., 1999; Ye and Sontheimer, 1999), although the intervening signaling pathway for how this might occur is unclear (Duan et al., 1999).

Expression levels of the transporters in culture are not representative of those *in situ*, nor are the subcellular vs. membrane distributions of the transporters equal across cultured systems or to those *in situ*. For example, primary cultures of astrocytes express very high levels of GLAST but very low levels of GLT-1 (Aronica et al., 2003), essentially opposite to the situation *in situ*. Co-culturing with neurons increases astrocytic expression levels of GLT-1

through soluble factors, but astrocytes often co-express neuronal transporters, and neurons express transporters sensitive to the GLT-1 specific antagonist DHK (Schlag et al., 1998; Gegelashvili et al., 2000). There is evidence that GLT-1 is not even functional in cell cultured systems, which would certainly be expected to affect its modulation and trafficking (Schlag et al., 1998). Furthermore, astrocytes cultured in the absence of neurons do not express mGluRs (Aronica et al., 2003) and likely other GPCRs. Finally, these studies often employ very long incubation times of very high concentrations of glutamate, which kills neurons in co-cultures (Schlag et al., 1998) and likely rapidly desensitizes receptors (if they are present), preventing an assessment of receptor-mediated regulation of transporter function (Duan et al., 1999).

Despite the large body of work using cell culture with confounding results, the hypothesis that synaptically released glutamate can modulate astrocytic glutamate uptake via activation of mGluRs on astrocytes has not been tested in a slice preparation or *in vivo*. We tested this hypothesis in an acute hippocampal slice preparation and the experiments are detailed in the following Chapters. Synaptically evoked glial transporter currents were demonstrated in a slice preparation for the first time in Bergmann glial cells of the cerebellum following parallel fiber stimulation (Clark and Barbour, 1997). Following this study, astrocytic transporter currents were reported in hippocampal slices following stimulation of Schaffer collaterals (Bergles and Jahr, 1997). A couple of subsequent studies used astrocytic currents as a sensor for neuronal glutamate

release (Diamond et al., 1998; Lüscher et al., 1998). It was also reported that astrocytic transporter currents are faster in hippocampal slices from adult rats compared to P12-P14 (Diamond, 2005). These efforts in hippocampal slices published between the years 1997 and 2005 standardized the hippocampal slice preparation for studying astrocytic transporter currents and helped us in designing our study.

1.7. Hippocampal Circuitry

The hippocampus is one of the most studied areas of the mammalian central nervous system due to its readily identifiable gross and histological structure and because of its fundamental role in some forms of learning and memory. The basic circuitry of the hippocampal formation has been known since the time of Ramon y Cajal, although details worked out by modern neuro-anatomists have contributed to our current understanding. Neurons in the entorhinal cortex give rise to the perforant path that projects through (perforates) the subiculum and terminates both in the dentate gyrus and in the CA3 field of the hippocampus. The dentate gyrus is the next step in the progression of connections, and it gives rise to the mossy fibers that terminate on the proximal dendrites of the CA3 pyramidal cells. The CA3 pyramidal cells, in turn, project heavily to other levels of CA3 as well as to CA1. The projection to CA1 is typically called the Schaffer collateral projection. CA1 pyramidal cells give rise to connections both to the subiculum and to the deep layers of the entorhinal

cortex. The subiculum also originates a projection to the deep layers of the entorhinal cortex. The deep layers of the entorhinal cortex, in turn, originate projections to many of the same cortical areas that originally projected to the entorhinal cortex. Thus, information entering the entorhinal cortex from a particular cortical area can traverse the entire hippocampal circuit through these excitatory pathways and ultimately return to the cortical area from which it originated. The transformations that take place through this traversal are presumably essential for enabling the information to be stored as long term memories (Johnston and Amaral, 2004).

1.8. Astrocytic Glutamate Uptake in Hippocampal CA1 Synapses

Perhaps the most studied synapses in the CNS are those that occur between Schaffer collaterals (SCs) originating from CA3 pyramidal cells and CA1 pyramidal neurons in the hippocampus. Much is known about this type of synapse, including its cytoarchitecture and its propensity for plasticity. Here, glial cells marginally envelop synapses and yet are responsible for most of the glutamate uptake (~ 80%). Of the five cloned glutamate transporters, three are found in the hippocampus. EAAT1 and EAAT2 are primarily expressed in glia, whereas EAAT3 is found in neurons. A splice variant of EAAT2 has also been found in some neurons, but its expression level is fairly low, and its function remains unclear. EAATs are found at a high density in the hippocampus, with an estimated concentration of 0.7–1.3 mM, and densities of 2,300 and 8,500

molecules per μm^2 for EAAT1 and EAAT2, respectively. EAAT3 is found in much lower concentrations, but accurate measurements have not taken place due to the lack of good antibodies for this protein (reviewed in Tzingounis and Wadiche, 2007).

It was thought until recently that blocking glutamate uptake does not affect α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA (N-methyl-D-aspartate)-receptor-mediated synaptic transmission at SC–CA1 synapses. However recent studies using highly potent glial-transporter blocker, (2S, 3S)-3-[3-[4-(trifluoromethyl)benzoylamino] benzyloxy]aspartate (TFB-TBOA), showed that the NMDA-receptor mediated EPSC's decay kinetics are significantly prolonged in the presence of TFB-TBOA. Changes were also detected in AMPA-receptor mediated EPSCs if glutamate-transporter block occurred when receptor desensitization was relieved with cyclothiazide (CTZ) (Tsukada et al., 2005). Though this study demonstrated that compromising glutamate uptake using pharmacological agents can shape neuronal currents, it is not known if a similar phenomenon operates under physiological conditions.

It has been estimated that glial cells contact less than half of SC–CA1 synapses (Ventura and Harris, 1999), raising the possibility that glutamate released from neighboring terminals might pool and spillover to adjoining synapses. Non-specific glutamate-uptake blockers significantly prolong the decay of NMDA-receptor EPSCs in conditions of high-frequency stimulation, but not when the stimulation is restricted to the activation of a few fibers, or in paired

recordings between a presynaptic and a postsynaptic cell (Arnth-Jensen et al., 2002) suggesting that glial glutamate transporters enhance synapse independence and limit the spillover of glutamate to neighboring synapses.

1.9. Glutamate Uptake and Neurological Disease

Glutamate transporter dysfunction may either be an initiating event or part of a cascade leading to cellular dysfunction and ultimately cell death (Maragakis and Rothstein, 2004). Direct evidence for the potential importance of glutamate transporters comes from the GLT1 knockout mouse. The absence of this astroglial transporter results in only 5% residual glutamate transport in GLT1 homozygous null mice. Histological and physiological changes were observed in the hippocampus with loss of neurons resulting in seizure activity and subsequent early mortality (50% die by 6 weeks of age) in these mice (Tanaka et al., 1997). Recently it has been shown that deficits in inhibition following compromised glutamate-glutamine cycle in reactive astrocytosis can lead to epilepsy (Ortinski et al., 2010). In amyotrophic lateral sclerosis, a mutation in SOD1 gene leads to a downregulation of GLT1 followed by neurodegeneration and paralysis (Maragakis and Rothstein, 2004). Amyloid precursor protein, thought to play a central role in Alzheimer's disease, has been shown to regulate astrocytic glutamate transporters (Masliah et al., 2000). Deficits in astrocytic glutamate transport have also been observed in Parkinson's disease, Huntington disease, Stroke, Ischemia and Glioma (Maragakis and Rothstein, 2004).

Recently it has been found that Ceftriaxone, a β-lactam antibiotic related to penicillin offers neuroprotection in models of ischemic injury and neuro-degeneration. The suggested mechanism for this neuroprotection is a reduction in glutamate mediated excitotoxicity by increasing the transcription of the GLT1 gene (Rothstein et al., 2005).

The different aspects of astrocytic glutamate transport discussed so far convincingly depict the importance of studying the regulation of this process to better understand its effects on physiological and pathological neuro-transmission. Not surprisingly, the summary statement of a workshop on the role of astrocytes in health and disease (NINDS, 2002) states that one of the main foci of future research on astrocytes should be to determine in greater detail the properties of glutamate transporters and their regulation *in situ*. This dissertation study caters to this need and the experiments towards this end based on a hippocampal slice model are the focus of the following Chapters.

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Chapter 2

Measuring Astrocytic Glutamate Uptake in a Mouse Hippocampal Slice Preparation and General Methods

2.1. Synaptic activation evokes a complex inward current in astrocytes

Glutamate uptake by astrocytes is coupled to the co-transport of a proton and three sodium ions and the counter-transport of a potassium ion. In addition to the transport of these ions, there is a thermodynamically uncoupled bidirectional anion transport, the significance of which is unknown (reviewed in Tzingounis and Wadiche, 2007). The net movement of charges associated with uptake of one glutamate molecule is represented in the equation below:

$$1\ \text{Glutamate}^- + 3\ Na^+ + 1H^+ - 1K^+ = \text{Influx of 2 positive charges}$$

This influx of two positive charges can be recorded as an inward current in patch-clamped astrocytes. When glutamatergic neurons are stimulated in slices, they release not only glutamate in the synapse but also potassium ions into the extracellular space. Astrocytes take up both the glutamate which spills out of the synapse and the potassium ions released by neurons (Bergles and Jahr, 1997; Bergles et al., 1999). Hence, the inward current measured in patch clamped astrocytes following synaptic activation is a complex inward current representing uptake of both glutamate and potassium. The current due to glutamate uptake is blocked by a non-selective glutamate transport blocker *threo*-β-Benzyloxyaspartic acid (TBOA). This TBOA sensitive transporter current has

faster rise and decay times compared to the TBOA insensitive component. The slower TBOA insensitive component has been demonstrated to be mainly due to potassium uptake in earlier reports (Bergles and Jahr, 1997).

2.2. Isolation of glutamate transporter currents from the total inward astrocytic current evoked by Schaffer collateral stimulation

As most of our experiments involved measuring transporter currents in stratum radiatum (s.r.) astrocytes, we sought to determine the minimum intensity needed to stimulate Schaffer collaterals to generate glutamate uptake mediated inward current in astrocytes. The rationale for using minimal stimuli is to release a low concentration of glutamate to minimize any spill over from the transporters to activate the metabotropic receptors on astrocytes. This stimulation intensity can then be used to study the effect of activation of metabotropic receptors on transporter currents as evoking transporter currents by itself will not activate endogenous metabotropic receptors on astrocytes.

A pilot experiment suggested that stimulation intensities below 50 µA were unreliable in evoking transporter currents. With a stimulation intensity of 75 µA we could reliably evoke transporter currents in astrocytes (Fig. 2.1). The amplitude of the current evoked using this stimulation intensity was in the 25 - 50 pA range. This evoked current, though smaller in amplitude, had a fast component due to glutamate transport which was blocked by the non-selective glutamate transport blocker TBOA (Fig. 2.2) just like the current produced by larger stimulation intensities. There was also a TBOA insensitive current with a

slower rise, thought to be mainly due to potassium uptake (Bergles and Jahr, 1997).

Application of TBOA for longer times or at higher concentrations in slices will increase the ambient glutamate concentration within the slice and will have unpredictable confounds. This prevents the use of TBOA or other transport blockers to isolate transporter currents in experiments aimed at measuring transporter currents repeatedly over 30 min. We employed a recently developed protocol (Diamond, 2005) which enabled us to isolate the glutamate transporter component without resorting to TBOA application. In this protocol, the K^+ currents are acquired in separate recordings and these are subtracted from the total current acquired in later experiments, thus isolating the glutamate transporter currents non-pharmacologically. The sequence of steps in this protocol is as follows: A 10 s electrophysiological recording (sweep) containing a single pulse stimulus was followed by another 10 s sweep with paired pulses. Stimulation pulses were 75 μA in amplitude and 200 μs in duration. This stimulation sequence was repeated continuously using the sequencing tool in the Clampex acquisition software. Sequential stimulation was stopped for brief moments to apply train stimulation or for monitoring cell health. Two such single pulse-paired pulse combinations were chosen at specific time intervals for isolating transporter currents from the total evoked currents. As the TBOA insensitive component (referred to as 'K^+ current' hereafter as it is mainly due to potassium uptake) had a slower rise and reached steady state ~70 ms (check) after the stimulation, it

could be fitted using a single exponential fit. The steady state of the total evoked current ~100 ms after the stimulation is entirely due to the K^+ current because it matches exactly the amplitude in the absence of TBOA. Hence, the amplitude of this K current can be easily determined by measuring the steady state current ~100 ms after stimulation. An estimate of the τ_{rise} of this slow rising K+ current was obtained from a set of slices following a 5 min TBOA (25 µM) application. A pilot experiment suggested to us that 25 µM TBOA is sufficient to completely block the fast transporter component as has been reported for 100 µM TBOA (Diamond, 2005). We then generated the slow rising K^+ current using Clampfit software by a standard single exponential fit using the τ_{rise} estimate and the steady state amplitude (measured ~100 ms after the stimulation). This enabled us to non-pharmacologically isolate the transporter currents in subsequent experiments. An example of this procedure is shown in Fig. 2.3 A&B. Surprisingly, we found that the K^+ current in slices from P12-P14 day old mice had a slower rise (mean τ_{rise} of 10.53 ms; n = 9 cells, S.D. = 1.93) compared to P15-P18 slices (mean τ_{rise} 5.74 ms; n = 8 cells, S.D. = 1.38) (Fig. 2.4). The τ_{rise} of P15-P18 slices is similar to that observed for p13 slices by (Diamond, 2005). Since in the Diamond (2005) study, the primary comparison was between juvenile and adult slices, it is possible that such a subtle difference in ages was not problematic in their experiments. Because of this difference in the τ_{rise} between the age groups, we used two different τ_{rise} estimates to generate the K^+

current based on the respective age groups for isolating the transporter component.

2.3. Minimal stimuli used to measure glutamate uptake could not exclude activation of astrocytic metabotropic receptors

We chose a stimulation intensity of 75 µA for our test pulses used to evoke transporter currents for measuring uptake as it was the lowest intensity with which we could reliably evoke a transporter current in our preparation (Fig. 2.1). We tested whether the glutamate released by this minimal stimulation activated endogenous astrocytic metabotropic glutamate receptors. Global calcium response of astrocytes was taken as a measure of astrocytic group I mGluR activation (Honsek et al.; Porter and McCarthy, 1996; Bowser and Khakh, 2004) . We also observed that astrocytes also respond with microdomain calcium elevations following glutamate release by afferent fibers. These responses have been found to occur using lesser stimulation intensities than described here. Detailed analysis of the different type of calcium responses is beyond the scope of this study.

The percentage of bolus loaded astrocytes responding with a global calcium response was calculated after various stimulation intensities. We found that the 75 µA stimulation intensity used for our test pulses did increase the percentage of cells exhibiting a global calcium response compared to basal levels (34% after 75 µA stimulation vs. 16% under basal conditions; % based on

total number of imaged cells). However, this percentage is significantly lower than the percentage of cells responding with a global calcium response following a 200 Hz train of 50 µA stimuli (68% of total cells imaged) (Fig. 2.5). As only global responses were measured in this experiment, it is possible that microdomain calcium responses might be produced at even higher percentages. So, it seems impossible to reliably evoke astrocyte transporter currents without simultaneously stimulating endogenous astrocytic metabotropic receptors. We controlled for this caveat in subsequent experiments by including additional experimental groups in which antagonists for the metabotropic glutamate receptors were included in the ACSF to prevent activation of endogenous metabotropic receptors on astrocytes during our test pulse stimulation.

2.4. General Methods

2.4.1. Hippocampal Slice Preparation

All experiments were done using hippocampal slices from 12-18 day old C57BL/6J (Jackson Laboratory) or the transgenic MrgA1 mice backcrossed at least 10 generations to the C57BL/6J background (Fiacco et al., 2007). Mice were decapitated following isoflurane anesthesia and the brains were isolated. Parasagittal hippocampal slices were then prepared in ice-cold, nominally Ca^{2+}-free saline containing (in mM): 125 NaCl, 2.5 KCl, 3.8 $MgCl_2$, 1 NaH_2PO_4, 26.6 $NaHCO_3$, and 25 glucose, bubbled with 5% CO_2-95% O_2. Subsequently slices were incubated at 35°C in oxygenated artificial cerebrospinal fluid (ACSF)

containing (in mM) 125 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.6 $NaHCO_3$, and 15 glucose, bubbled with 5% CO_2-95% O_2. Following the incubation period at physiological temperature, slices were allowed 15 min to equilibrate to room temperature and then transferred to a recording chamber and continuously superfused with oxygenated, room temperature ACSF.

2.4.2. Electrophysiology Instrumentation

Either a Multiclamp 700B + Digidata 1440A or Axopatch 200B + Digidata 1320 amplifier-digitizer combination (Molecular Devices, Sunnyvale, CA) was used for patch clamp experiments. Individual astrocytes in CA1 stratum radiatum were identified by their location, size, and morphological characteristics and patch clamped using a borosilicate patch pipette (7-8 MΩ) filled with (in mM): 130 K-gluconate, 4 $MgCl_2$, 10 HEPES, 10 glucose, 1.185 Mg-ATP, 10.55 phosphocreatine, and 0.1315 mg/ml creatine phosphokinase, pH 7.3 by KOH (standard astrocyte internal solution). Current signals from the patch electrode were low-pass filtered at 2 kHz and digitized at 100 kHz. During recordings, drugs were bath-applied using an electronic valve controller (Warner Instrument, Hamden CT).

2.4.3. Patch clamping passive stratum radiatum astrocytes and placement of the stimulating electrode

Astrocytes in the CA1 stratum radiatum were identified by their complex morphology using Differential Interference Contrast (DIC) optics. After visually

locating an astrocyte for patch clamping, a glass monopolar stimulating electrode was placed approximately 150 µm towards the CA3 side from the astrocyte. The stimulating electrode was constructed as follows: Two chlorided silver wires - one inside a glass micropipette of resistance ~ 3 mΩ in contact with ACSF and the other outside the glass pipette fixed using soft clay and touching the bath ACSF - were connected to a stimulus isolator (Warner Instruments, Hamden, CT). Pulse duration was set to 200 µs. The stimulating electrode was placed slightly below (20-30 µm) the optical plane of the chosen astrocyte. The schematic diagram in Figure 2.6 depicts a hippocampal slice with the stimulating electrode and patch pipette in place. After the stimulating electrode was in place, the astrocyte was patch clamped and the passive current profile was obtained using 20 mV steps ranging from -180 mV to +80 mV (14 steps total). Only cells exhibiting a passive electrophysiological phenotype were used for further study (Fig. 2.7), as these are the astrocyte subtype known to take up glutamate (Matthias et al., 2003). A test pulse of -5 mV was included after each voltage step in order to monitor changes in access resistance, a standard procedure in whole-cell patch clamp studies.

2.4.4. Astrocytic Calcium Imaging

For measuring astrocytic calcium responses, astrocytes in the stratum radiatum were bolus loaded with the green calcium indicator dye, Oregon Green Bapta-1 (OGB-1). The procedure consisted of lowering a pressure pipette of low

resistance (~1.3 mΩ) filled with ACSF containing 200 μM OGB-1-AM (acetoxymethyl ester) into the slice to ~50 μm depth and leaving it in place for about 1 min and repeating it at two more neighboring locations such that ~ 4 -10 astrocytes are filled with the dye (Nimmerjahn et al., 2004; Sullivan et al., 2005; Garaschuk et al., 2006). Astrocytes in the slice were labeled with Sulforhodamine 101 (SR-101) prior to bolus loading for verification (Nimmerjahn et al., 2004). Pre-labeling was done in a 12 well culture plate by submerging the slices in oxygenated ACSF (650 μl) containing 1 μM SR-101. Dye-filled astrocytes were visualized using an Olympus confocal microscope. OGB-1 was visualized using an excitation laser of 488 nm and the emission spectrum in the FITC range was collected for analysis. SR-101 was detected using a 555 nm excitation laser and emission was in the rhodamine spectrum (Fluoview 1000 software, Olympus Corporation). Photomultiplier tubes were set with an aperture size of 550 μM (3.4 x Airy Disc). Changes in fluorescence intensity over time were recorded by placing 'Regions of Interest' analysis boxes (ROIs) over the somata of astrocytes, using Olympus Fluoview 1000 software. Increase in the average fluorescence intensity over baseline fluorescence (F/F_0) within each ROI indicated increases in Ca^{2+} concentration (Takahashi et al., 1999).

2.4.5. Statistical analysis

Comparisons between groups were done using Student's independent *t* test. Most of the experiments involved a simple repeated measures design with

repeated current measurements from the same cell over time. Repeated Measures - Analysis of Variance (RM-ANOVA) is an appropriate parametric test for analyzing data from this kind of experimental design if there are no missing samples (David Rowland, 1991). In our experiments, the sample size (n) always decreased over the course of the experiment due to changes in access resistance. Using RM-ANOVA, in such cases, reduces the 'n' available for analysis to the number of cells which lasted until the last measurement time point (30 min). In order to better represent the 'n' in acquired data, Student's paired *t* test was used to compare measurements from the same cell over time and $p < 0.05$ are indicated in the graphs. Such analysis based on multiple comparisons, despite creating a risk of chance level significance (Type I error – false positive), helped us mitigate 'not completely at random' missing samples. Multiple comparisons using paired *t* test gives adequate control over Type I error if Dunn critical values are used instead of regular *t* distribution values. An 'α' value of 0.01 using a *t* distribution translates to 0.04 (4 x 0.01) for 4 comparisons if Dunn critical values are used (Toothaker, 1991; Myra L. Samuels, 2003). As the usual number of comparisons in our experiments were 4, differences significant at the 99% ($\alpha = 0.01$) using paired *t* test can be considered as significant at least at the 95% ($\alpha = 0.05$) confidence interval if the conservative Dunn critical values are used. It should also be noted that multiple *t* tests limit only the effect of a reduction in sample size at later time points on an earlier time point, but still suffer from the loss in statistical power due to reduced sample size at the

measured time point. Imputational alternatives to missing data points are complicated by the ‘not completely at random’ nature of the missing data and hence not applied in our analysis.

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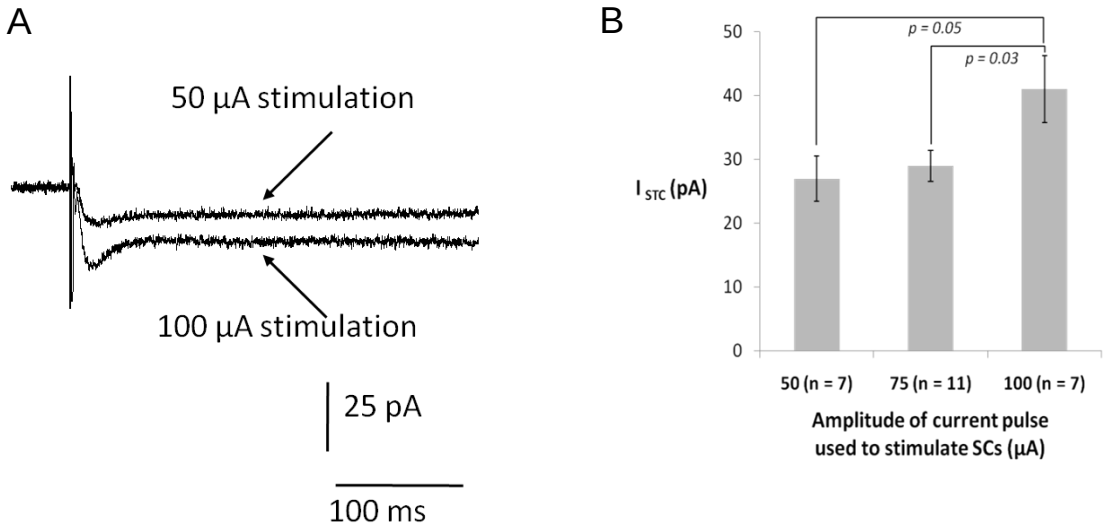

Fig. 2.1: Stimulation intensity threshold for evoking astrocytic glutamate transporter currents: A) Averaged astrocytic inward currents from different slices ('n' indicated in the histogram in B) produced by 50 and 75 µA stimulation of Schaffer collaterals. B) Histograms representing averaged peak amplitude of the inward current evoked by three different stimulation intensities. 'n' in parentheses represents number of cells. Though 50 and 75 µA stimulations evoked transporter currents of equal amplitude, there was greater variability with 50 µA stimulation. There were also instances were 50 µA stimulation did not evoke a current (not represented here).

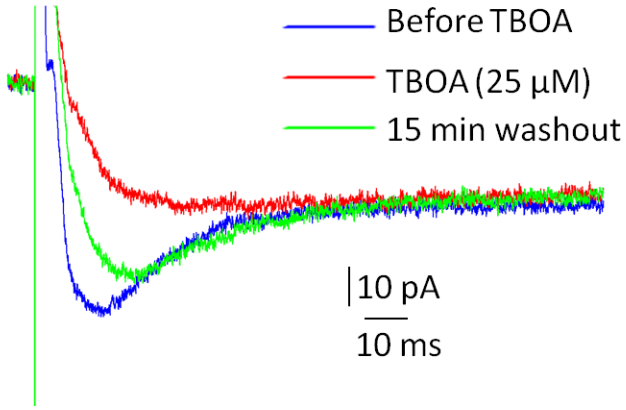

Fig. 2.2: Fast component of the Schaffer collateral stimulation evoked astrocytic current is due to glutamate transport: Bath application of 25 µM of the non selective glutamate transport blocker DL-threo-Benzyloxyaspartic acid (TBOA) for 5 min almost totally inhibited the fast component of the current evoked using a 75 µA pulse. We used a concentration of 25 µM for TBOA as it was sufficient to produce a block similar to that described in the literature using 100 µM TBOA. Wash out 25 µM TBOA also took shorter time (~ 15 min) and it was possible to re-evoke the transporter current after washing out.

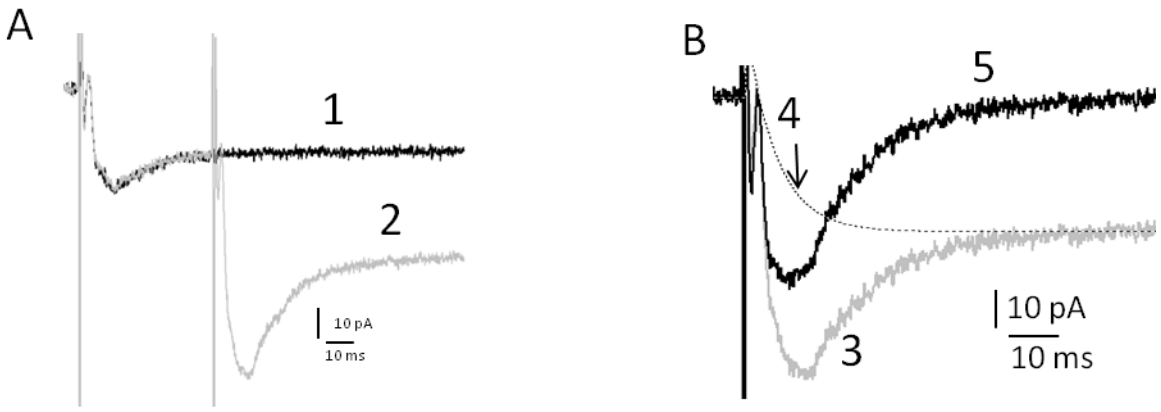

Fig 2.3: Protocol for isolating glutamate transport component from the total evoked current: A) Astrocytic currents evoked using a single 75 µA pulse (black trace: 1) and a pair of pulses (20Hz) (gray trace: 2) to the Schaffer collaterals. B) Current evoked by the single pulse (trace 1 in A) was subtracted from that evoked by the paired pulse protocol (trace 2 in A) to isolate the total facilitated current which has a greater glutamate transport component due to presynaptic facilitation of glutamate release (gray trace in B: 3). Potassium current generated using τ-rise estimated from a different set of slices in the presence of TBOA and the amplitude 100 ms after stimulation (dotted trace: 4) was subtracted from the facilitated current (3) to isolate the current specifically due to glutamate transport (black trace: 5)

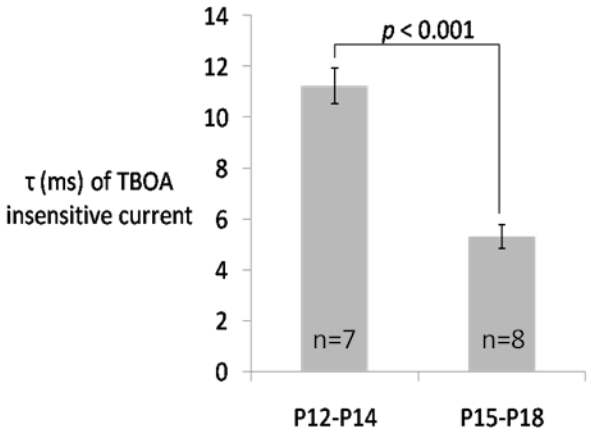

Fig. 2.4: Rise time of K^+ current: TBOA insensitive K^+ currents had a faster τ rise between P12-P14 compared to P15-P18.

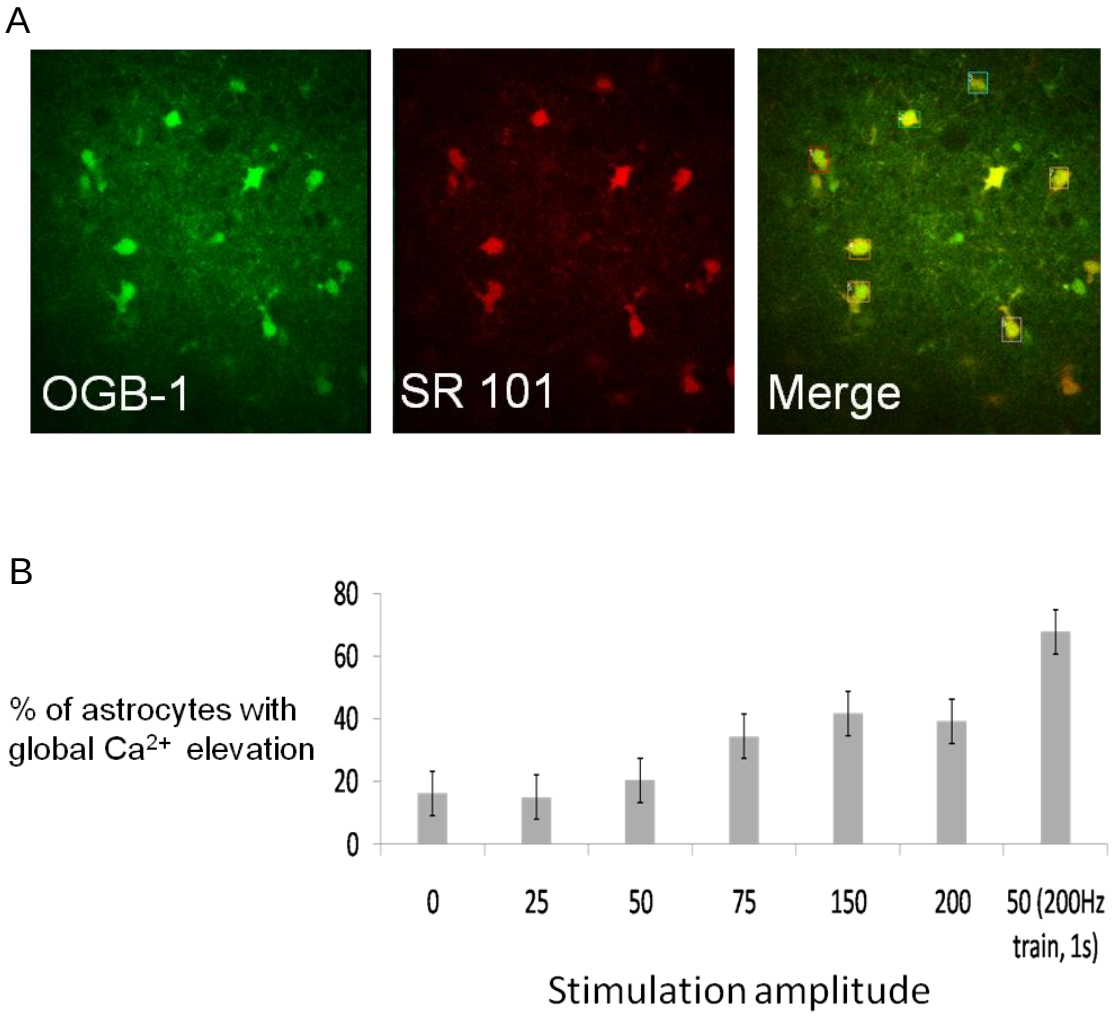

Fig. 2.5: SC stimulation intensity used to measure transporter currents induces Ca^{2+} responses in astrocytes: Astrocytic Ca^{2+} responses following various stimulation intensities were measured in slices bolus loaded with the calcium indicator dye Oregon Green BAPTA (OGB -1). A) Identity of the s.r. cells loaded with OGB-1 verified using the astrocyte specific marker SR 101. B) % of astrocytes exhibiting a global Ca^{2+} elevation is plotted against the amplitude of the current used to stimulate Schaffer collaterals. 75 µA stimulation intensity did increase the percentage of cells exhibiting global Ca^{2+} elevation compared to baseline levels (n = 25 cells, 4 slices).

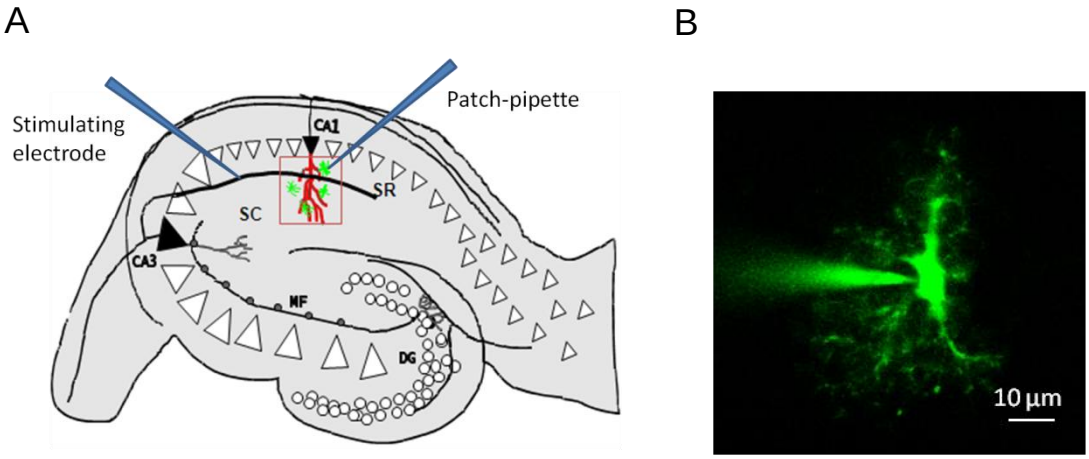

Fig. 2.6: Hippocampal slice model: A) Schematic sagittal section of hippocampus showing astrocytes in CA1 stratum radiatum (green) surrounding synapses formed by Schaffer collaterals on CA1 pyramidal neurons. B) Example of a patch clamped s.r. astrocyte filled with OGB-1

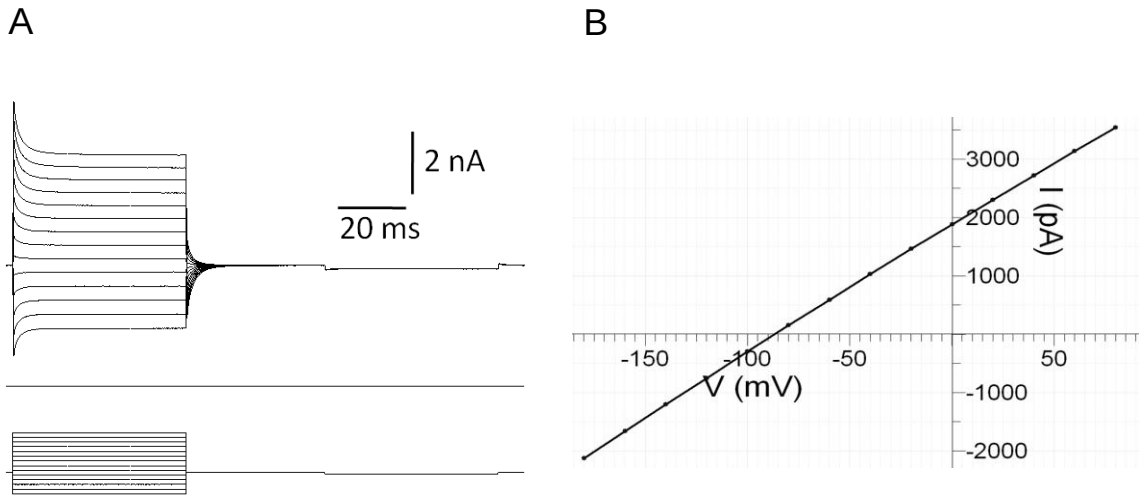

Fig. 2.7: I-V Plot from a passive astrocyte: A) Voltage steps (20 mV) from -180 mV to +80 mV do not produce any voltage dependent currents in astrocytes.
B) I-V Plot demonstrates the linear I-V relationship. This electrophysiological profile was used in the beginning of each recording to verify passive astrocytes.

Chapter 3

Tetanic Stimulation of Schaffer Collaterals Potentiates Astrocytic Glutamate Uptake by an mGluR – PKC Dependent Mechanism

Abstract

The involvement of astrocytic metabotropic glutamate receptors (mGluRs) and protein kinase C (PKC) in the modulation of glutamate transport has been tested in culture preparations with confounding results. The experiments in this chapter use an acute mouse hippocampal slice preparation to test the hypothesis that synaptically released glutamate modulates glutamate uptake by an mGluR-PKC dependent mechanism. Tetanic high frequency stimulation (HFS) of Schaffer collaterals was used to activate endogenous astrocytic mGluRs. HFS potentiated astrocytic glutamate uptake as evidenced by an increase in the amplitude of the synaptically evoked transporter currents (STCs) without accompanying changes in kinetics. A similar increase in the amplitude of the STCs following HFS was not observed in the presence of group I mGluR antagonists or in the presence of a PKC inhibitor, suggesting that HFS induced potentiation of glutamate uptake is mGluR-PKC dependent. HFS also enhanced the amplitude of the potassium uptake induced slow inward K^+ current, which might be a passive reflection of increased extracellular K^+ following HFS.

Introduction

It is well-established that astrocytes express group 1 metabotropic glutamate receptors (mGluRs) which can be stimulated by neuronal activity (Porter and McCarthy, 1996; Wang et al., 2006b; Schummers et al., 2008). While there was a speculation as early as 1996 (Porter and McCarthy, 1996) that activation of astrocytic glutamate receptors might be involved in regulating glutamate uptake, the hypothesis has not been tested in a slice preparation or *in vivo.* Up regulation of glutamate uptake by PKC mediated phosphorylation has been reported in HeLa cells transfected with GLT-1. A study using culture preparation also concluded that astrocytic glutamate uptake is up regulated following glutamate application by a mechanism dependent on transport *per se* and independent of metabotropic receptors (Duan et al., 1999). However, the procedure employed in this study to measure glutamate uptake renders the conclusion questionable. Similar studies on cultured cell types have generated a large body of work which at best provides a muddled picture of whether or not astrocyte glutamate transport is acutely modulated by GPCRs or their signaling molecules (discussed in Chapter 1). One of the well known culture based study in the field reported calcium dependent glutamate release by astrocytes following activation of astrocytic Gq GPCRs (Parpura et al., 1994). However, activation of astrocytic Gq GPCRs in hippocampal slices failed to release glutamate (Fiacco et al., 2007), questioning the use of culture preparations for studying the physiological role of astrocytes in neurotransmission. Because of contradictory

reports similar to these, the physiological role of astrocytic Gq GPCR activation remains controversial (reviewed in Agulhon et al., 2008).

As synaptically released glutamate can have multiple targets on astrocytes – primarily glutamate transporters and metabotropic glutamate receptors, the hypothesis that glutamate uptake might be modulated by activation of astrocytic metabotropic glutamate receptors has remained an alluring hypothesis but never tested in a slice preparation until this study. The first experiment described in this Chapter (3.1) demonstrates that astrocytic glutamate uptake is potentiated following tetanic stimulation of Schaffer collaterals. Evidence from Purkinje neurons of the cerebellum and cultured astrocytes suggest that mGluRs and the downstream signaling molecule protein kinase C (PKC) are involved in the regulation of glutamate transport (Casado et al., 1993; Aronica et al., 2003; Shen and Linden, 2005; Susarla and Robinson, 2008). Metabotropic glutamate receptors fall into two major families - Gq and Gi coupled (Alexander et al., 2007). Of these two types of mGluRs, the Gq subtype has received wider attention as activation of Gq receptors can be monitored by imaging [Ca^{2+}] and it is this type of mGluRs (group I) whose involvement in modulating glutamate uptake is studied here. Experiments 3.2 and 3.3 are aimed at testing if group I mGluRs and PKC are involved in the tetanic stimulus induced potentiation of astrocytic glutamate uptake. Results strongly suggest that astrocytic group I mGluRs and PKC are necessary for HFS induced potentiation of glutamate uptake.

Results

Experiment 3.1: Effect of tetanic high frequency stimulation of Schaffer collaterals on astrocytic glutamate uptake

We tested the effect of tetanic high frequency stimulation (HFS) (1 s, 200Hz train consisting of 200 µs, 75 µA pulses) on glutamate uptake. Test STCs to measure glutamate uptake were evoked as described in Chapter 2. STCs selected before and at 2, 5, 15 and 30 min after HFS were used for comparison. We found that the peak amplitude of the STCs increases gradually after the HFS and becomes significantly larger (161%, $p = 0.016$; $n = 9$ cells; S.D. = 59.9) at 30 min after the HFS (Fig. 3.1). We could not record longer than 30 min following HFS due to cell viability and access resistance issues in whole cell patch clamp experiments, which have also been described by other groups (Diamond et al., 1998; Lüscher et al., 1998).

Similar to these findings on glutamate transport by hippocampal astrocytes, neuronal glutamate transport in Purkinje neurons of the cerebellum has been shown to undergo long term potentiation following a tetanic stimulation protocol (Shen and Linden, 2005). Two earlier studies in hippocampal slices aimed at deciphering the locus of neuronal long term potentiation (LTP) to the presynaptic or postsynaptic site measured astrocytic transporter currents following a 100 Hz stimulation of Schaffer collaterals (Diamond et al., 1998; Lüscher et al., 1998). Both studies provided identical results that astrocytic transporter currents do not undergo any change after a 100 Hz stimulation of Schaffer collaterals, therefore providing evidence that the locus of LTP is

postsynaptic. While it has been concluded that astrocytic transporter currents just mimic the amount of glutamate released by neurons, it is possible that the use of kynurenic acid in these studies might have diminished the effect on the astrocytic STCs. Both of these studies measured astrocytic glutamate transport in the presence of kynurenic acid at very high concentrations which can activate GPR 35, a Gi GPCR (Wang et al., 2006a). A recent study has demonstrated the expression of GPR 35 in primary glial cell cultures (Cosi et al., 2011). Hence, it is possible that the above two studies did not find an increase in glutamate uptake as a Gi receptor was activated simultaneously with Gq mGluRs. It is also possible that the 100 Hz train may not have been sufficient to saturate the transporters which are present at a very high density (Lehre and Danbolt, 1998). In our studies we used 200 Hz train stimulation. We find that following a 200Hz train, the currents evoked in astrocytes plateau about 75 ms from the first stimulation pulse unlike a 100 Hz train stimulation which did not produce this plateau (Fig. 3.2). It is interesting to note that the duration (~75 ms) it takes for the STCs to plateau during the 200 Hz train is almost the same as the cycling rate of transporters (13/s = 1/77 ms) at a saturating concentration of glutamate (Otis and Jahr, 1998). This match in the time to plateau with the cycling rate supports the idea that the transporters are saturated following a 200 Hz train thus necessitating an increase in uptake capacity to keep up with increased glutamate release.

Experiment 3.2: Role of group I mGluRs in HFS induced potentiation of glutamate uptake

Our initial hypothesis was that glutamate diffusing out of the synaptic cleft is leading to the potentiation of transporter current amplitude after HFS by activating astrocytic mGluRs. Activation of group I mGluRs of CA1 astrocytes following stimulation of Schaffer collaterals with train stimuli has been observed by other groups (Honsek et al.; Porter and McCarthy, 1996; Bowser and Khakh, 2004). To test if group I mGluRs mediate the potentiation of transporter current amplitude, we repeated experiment 1.1 in the presence of the group I mGluR antagonists (2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 10 µM and (*S*)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), 100 µM) in the ACSF. In the presence of these group I mGluR antagonists, we did not observe any significant difference between the amplitude of the STCs measured before and at any of the measured time points after the HFS (Fig 3.3). As HFS potentiated STC amplitude in the absence but not in the presence of group I mGluR antagonists, it can be concluded that group I mGluR activation is necessary for HFS induced potentiation of STC amplitude. This observation contradicts the interpretations of an earlier cell culture study (Duan et al., 1999) in which a glutamate transporter dependent mechanism of stimulation of glutamate uptake was postulated. A detailed reasoning for this apparent contradiction is provided in the discussion. We also tested if group I mGluR antagonists are capable of blocking calcium responses evoked by HFS. Though the antagonists were able to completely block calcium responses evoked by a

group I mGluR agonist, dihydroxyphenyl glycine (DHPG, 50 µM), HFS induced calcium responses were inhibited only partially. In the presence of group I mGluR antagonists, there was a 30% reduction in the area under the curve of HFS induced normalized [Ca^{2+}] responses (Fig 3.4). An earlier study on the role of P2Y receptors on astrocytes also reported that the group I mGluR antagonists inhibit Schaffer collateral induced astrocytic calcium responses only partially and adding P2Y receptor antagonists led to a greater reduction in the calcium responses (Bowser and Khakh, 2004).

Experiment 3.3: Role of PKC in HFS induced potentiation of glutamate uptake

Activation of astrocytic group I mGluRs can trigger different signaling mechanisms within the cell. Of these, PKC might be a likely downstream candidate involved in the potentiation of transporter currents. PKC dependent phosphorylation of the neuronal glutamate transporters leading to an increase in the amplitude of the transporter current has been observed earlier (Shen and Linden, 2005). Studies based on cultured cell lines have provided conflicting results as to the effect of PKC activation on glutamate transport. Phorbol ester, an activator of PKC has been shown to stimulate glutamate transport in HeLa cells transfected with the astrocytic glutamate transporter protein GLT-1 (Casado et al., 1993). In contradiction, PKC activation decreased the cell surface expression of GLT-1 in a glioma cell line (Susarla and Robinson, 2008). That there are different isoforms of PKC and different stimulation protocols might

activate them differently might be a likely reason for this contradiction. Despite the conflicting results from culture studies, we sought to determine if inhibiting PKC intracellularly will prevent the HFS mediated potentiation of glutamate transporter current in acute hippocampal slices. We included an inhibitor of PKC (PKC 19-36, 10 μM) in the patch pipette solution and allowed it to diffuse for at least 15 min before recording STCs. PKC 19-36 has a molecular weight of 2.15 kDa and so this additional 15 min duration was provided to help fill the smaller astrocytic compartments with the inhibitor. In the presence of the PKC inhibitor, we did not observe any increase in the amplitude of the transporter currents following HFS (Fig 3.5), suggesting that PKC is necessary for HFS induced potentiation of glutamate uptake. Surprisingly, we also observed at 2 min and 5 min after the HFS that there was a small but significant decrease (~20%) in the amplitude of the STCs. This may be a consequence of an imbalance between the glutamate mediated signaling pathways which are dependent and independent of PKC. There was no significant difference in the amplitude of the transporter current at subsequent time points.

Effect of HFS on the kinetics of STCs

The kinetics of the normalized STCs remained unaffected after the HFS both in the absence and presence of mGluR antagonists (Fig. 3.6). The 20-80% rise time of the isolated transporter current was 1.79 ms (n = 16; S.D. = 0.7) and is in agreement with an earlier study (Diamond, 2005). The normalized negative

rise slope had a mean of 0.38 (n = 16; S.D. = 0.13). The decay τ ranged from 12.2 to 18.7 ms with a mean of 15.78 ms (n = 16; S.D. = 4.59). This decay time is similar to that observed for adult slices but approximately 4-5 ms longer than that of P12-P14 slices in the Diamond (2005) study. It is possible that this difference arises from the inclusion of a wider postnatal age group (P12-P18) in our study. Long Term potentiation of glutamate transport reflecting as an increase in amplitude without changes in kinetics has been shown in Purkinje neurons of the cerebellum following tetanic stimulation of the afferent climbing fibers (Shen and Linden, 2005). HFS in the presence of PKC inhibitor, led to a faster rise of the glutamate transporter current at 5 min following the 200 Hz train (Figs. 3.6B & C) indicated by a significant decrease in rise time (mean of 1.48 ms and 1.27 ms at before and 5 min after the HFS respectively; p = 0.0006) and a steeper normalized negative slope (mean of 0.43 and 0.50 at before and 5 min after the HFS respectively; p = 0.0005). There was no significant difference in the decay τ following HFS in the presence of the PKC inhibitor (Fig. 3.6A). The faster rise of transporter currents following HFS in the presence of PKC inhibitor may be resulting from a shift in the balance among glutamatergic signaling mechanisms which are tied or not to PKC activation within astrocytes.

Effect of HFS on K^+ current

To determine the effect of HFS on K^+ current, we measured the steady state current amplitude 100 ms after the test pulse used to evoke the STCs. This

time point was chosen because the glutamate transporter currents have returned to baseline after 75 ms and any remaining current is due to K^+ uptake. There was a significant increase in K^+ current amplitude at all measured time points (2, 5, 15 and 30 min) after the HFS. However, in the presence of group I mGluR antagonists the K^+ current amplitude showed significant increase only at 2 min after the HFS. This might be because at 2 min after HFS the extracellular K^+ might still remain elevated and the increase in K^+ observed might be a reflection of increased extracellular K^+. In the presence of the PKC inhibitor, HFS did not affect the amplitude of the K^+ current (Fig. 3.7).

Discussion

The main finding in this study is that tetanic HFS of glutamatergic Schaffer collaterals potentiates glutamate uptake in stratum radiatum astrocytes in the mouse hippocampal slice preparation. The potentiation is reflected as an increase in the amplitude of the isolated transporter currents without accompanying changes in kinetics. We also found that activation of group I mGluRs and PKC is necessary for the HFS induced potentiation of glutamate uptake. Our results are similar to the mechanism described for potentiation of the neuronal glutamate transporter EAAT4 in Purkinje neurons of the cerebellum following tetanic stimulation of the climbing fibers (Shen and Linden, 2005). One of the very early papers on the astrocytic glutamate transporter, GLT-1 also suggested that phosphorylation of GLT-1 transfected in HeLa cells increases

glutamate uptake (Casado et al., 1993). However a recent report from a glioma cell line transfected with GLT-1 came to the opposite conclusion that phosphorylation of GLT-1 decreases cell surface expression (Susarla and Robinson, 2008). Another study from a reactive gliosis like culture preparation (Aronica et al., 2003) reported that activation of group I and II mGluRs, respectively, down and up-regulated the expression of astrocytic glutamate transporters - the opposite of what our results suggest. Culture-based studies exploring the regulation of glutamate transporters fail to note that astrocytes in culture exhibit very different properties as compared to *in situ*. The main type of transporter expressed by astrocytes in pure culture is GLAST (Gegelashvili et al., 1997) whereas it is mainly GLT-1 *in vivo* (Lehre et al., 1995). Also the glioma cell line used in most culture studies has an intrinsic expression of EAAC1, the neuronal transporter. The astrocytic transporter, GLT-1 is usually transfected in this cell line (Robinson, 2006). This creates a very artificial situation wherein neuronal and astrocytic transporters are co-expressed in the same cell and might potentially confound interpretations. Another potential but controversial confound is the physiological relevance of calcium dependent glutamate release by astrocytes in culture (Agulhon et al., 2008).

The faster rise kinetics of STCs in the presence of PKC inhibitor following HFS is unexpected. Astrocytes, in addition to group I mGluRs, also express group II mGluRs (Lavialle et al., 2011), which mainly signal by decreasing cAMP production (Conn and Pin, 1997). Inhibition of PKC and simultaneous releasing

of massive amounts of glutamate by HFS may shift the balance in favor of group II mGluRs. The cyctosolic levels of cAMP determine the activity of Protein Kinase A (PKA) (Graves and Krebs, 1999). Studies in cultured cells agree that PKA downregulates astrocyte glutamate uptake (Gegelashvili et al., 2000; Aronica et al., 2003; Adolph et al., 2007; Rath et al., 2008). Stepped up group II mGluR signaling in the presence of the PKC inhibitor may decrease cytosolic cAMP and PKA activity leading to disinhibition of glutamate transporters resulting in faster kinetics. The underpinnings of how this imbalance in the intracellular signaling machinery translates to changes in kinetics of the transporter currents can be a subject of future experiments.

Though our results firmly support our hypothesis that the potentiation of astrocytic glutamate transporter current amplitude following HFS is mGluR-PKC dependent, an earlier study in a culture preparation (Duan et al., 1999) suggested a mechanism dependent on glutamate transporters *per se*. Duan et al. (1999) observed that replacing sodium in the glutamate containing pre-incubation culture medium abolished the glutamate induced increase in glutamate uptake. They also observed that *trans*-1,3-dicarboxylic acid (tACPD), a non-selective mGluR agonist, stimulated glutamate uptake following pre-incubation in a glutamate containing medium and the stimulation was abolished if sodium was replaced in the medium. They interpreted that glutamate and tACPD were unable to stimulate glutamate uptake following incubation in a Na^+-free pre-incubation medium containing glutamate because the transporters were not taking up

glutamate and tACPD, preventing transporter induced stimulation of glutamate uptake. However, they underestimated the effect of preventing astrocytic glutamate uptake. Preventing glutamate uptake in a glutamate containing pre-incubation medium will lead to persistence of glutamate in the extracellular culture medium causing desensitization of all glutamate receptors including mGluRs. What combination of effects of such a prolonged activation of astrocytic receptors will produce in cultured cells remains speculative. Perhaps more important are subsequent observations that astrocytes cultured in the absence of neurons do not even express mGluRs, fully preventing an assessment of the contribution of astrocytic mGluRs to modulation of glutamate uptake (Aronica et al., 2003). For these reasons, it is not surprising that when Duan et al. measured glutamate uptake following a 60 min pre-incubation, glutamate and tACPD application did not potentiate uptake. Under these highly artificial culture conditions it is possible that the astrocyte glutamate transporters present - almost exclusively GLAST - take on some auto-regulatory properties. Another argument in the study was that D-aspartate, another substrate for glutamate transporters which cannot bind to astrocytic mGluRs, was able to potentiate glutamate uptake and hence the potentiation is by a transporter mediated mechanism. The authors failed to note that they applied glutamate to the culture medium for 7 minutes to measure glutamate uptake induced by D-aspartate, duration sufficient for glutamate to activate mGluRs if they are present. Hence, it is inconclusive if the effect of D-aspartate is due to glutamate application or due to D-aspartate itself.

The authors also ignored this 7 min glutamate application to measure glutamate uptake in their interpretation of a result showing that application of a non-selective group I mGluR antagonist, MCPG, during the preincubation period failed to prevent potentiation of glutamate uptake. While MCPG was applied during the pre-incubation period, it wasn't applied during the 7 min period when glutamate uptake was measured by applying glutamate. Due to these concerns regarding the interpretation of results in the Duan et al. (1999) study, it is difficult to compare their results to ours in intact tissue where astrocytes express mGluRs and functional GLT-1 transporters. Our data strongly suggest that mGluRs and PKC are necessary for glutamate mediated potentiation of astrocytic STCs negating a mechanism dependent on stimulation of the transporters themselves.

We also found that the group I mGluR antagonists used in our experiments only partially inhibited the HFS induced astrocytic calcium responses. Bowser and Khakh (2004) also reported that the antagonists of group I mGluRs only partially inhibit Schaffer collateral stimulation-induced astrocytic $[Ca^{2+}]_i$ elevations. Given the insufficiency of the group I mGluR antagonists in preventing HFS induced $[Ca^{2+}]_i$ elevations, the dependence of group I mGluR activation for the potentiation of astrocytic glutamate uptake remains questionable. Our results so far have established that group I mGluR antagonists are necessary for HFS induced potentiation of astrocytic glutamate uptake. The experiments detailed in Chapter 4 test the sufficiency of Gq GPCR activation for potentiating glutamate uptake.

In addition to the potentiation of STC amplitude following HFS, we also observed a significant increase in the amplitude of the K^+ current following HFS at all measured time points. It is tempting to conclude that this observed increase in K^+ current amplitude is also mGluR dependent *in toto*, as we saw a similar increase only at one of the measured time points (2 min) after the HFS in the presence of group I mGluR antagonists. However, we need to exercise caution in such an interpretation as it is possible that inducing LTP induction might be contributing to the observed increase in K^+ current. Enhanced astrocytic potassium uptake following LTP induction using a 100 Hz, 1s stimulus has been demonstrated to be a passive reflection of increased extracellular [K^+] (Ge and Duan, 2007). While we did not test the effect of inhibiting group I mGluRs on HFS induced LTP, it is possible that group I mGluR antagonists will affect LTP induction (Balschun et al., 1999). In such a scenario, the amplitude of post-synaptic action potentials and the amount of extracellular K^+ available for astrocytic uptake will remain the same before and after the HFS in the presence of mGluR antagonists. K^+ uptake - unlike glutamate uptake - will be greatly affected by LTP induction as the source of glutamate is presynaptic whereas K^+ is released by both the presynaptic and postsynaptic neurons in the milieu of the same astrocyte. Notwithstanding this, we find an increase in K^+ current amplitude following stimulation of astrocyte-specific Gq GPCRs (Chapter 4) suggesting intrinsic regulation of K+ uptake by astrocytes independent of the passive increase observed after LTP-inducing protocols.

The effect of PKC inhibitor on K^+ current is most likely dependent on a mechanism intrinsic to astrocytes as the water soluble inhibitor was delivered via the patch-pipette into the astrocyte. The likely explanation for the PKC inhibitor preventing HFS induced enhancement of potassium uptake by astrocytes, despite an increase in extracellular [K^+], is a reduction in the astrocytes uptake capacity for [K^+]. This reasoning also suggests that PKC is involved in maintaining the ambient potassium uptake tone and inhibiting PKC reduces this basal K^+ uptake reflected as an absence of enhancement of K^+ current following HFS-LTP induced elevated extracellular [K^+]. Another possibility is that activation of mGluRs and inhibiting the downstream signaling molecule PKC will recruit a different signaling pathway resulting in reduced K^+ uptake.

Transporter current amplitude can be increased by any of the following mechanisms: 1) Insertion of more transporters into the membrane from a readily insertable local pool or insertion of newly synthesized transporters; 2) Increasing the catalytic efficiency of the transporters; 3) Redistributing the existing transporters near sites of glutamate release; 4) Turning on some of the transporters already present on the membrane assuming there are silent transporters whose activation is recruited depending upon need. Considering the temporal scale of the potentiation described here and the involvement of PKC, insertion of more transporters onto the membrane from a readily insertable pool seems to be the likely mechanism. Synthesis of new transporters can be ruled out, as synthesis and transport of new proteins and insertion into the membrane

is thought to take hours. Redistribution of transporters, turning silent transporters on and changing catalytic efficiency, though possible mechanisms for rapid potentiation of glutamate transport capacity are difficult to test in slice preparations. In the future, it will be very informative to test which of these mechanisms is/are involved in HFS induced potentiation of glutamate uptake. It will also be useful to determine if decreasing the level of synaptically released glutamate results in decreased glutamate uptake; i.e. that these effects are bidirectional depending upon the amount of glutamate released by neurons.

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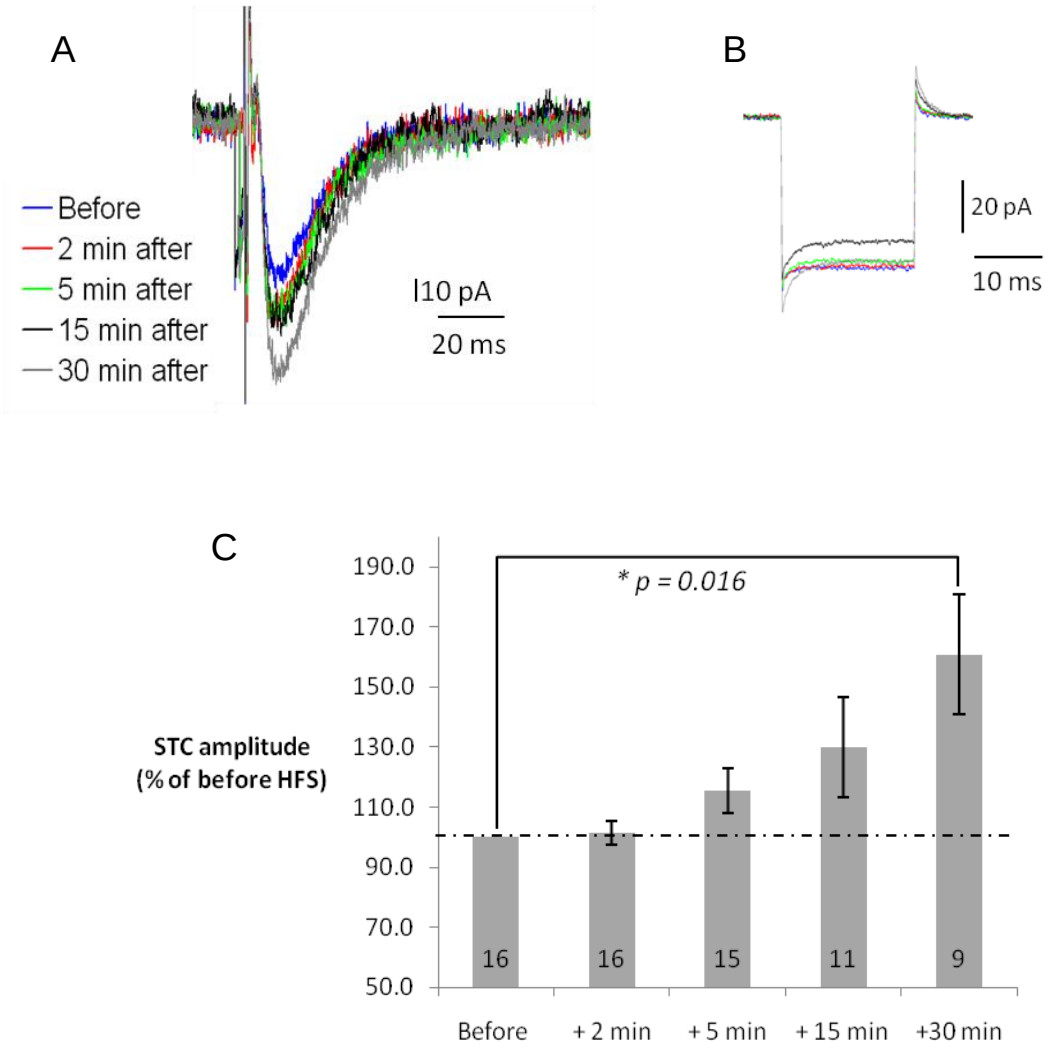

Fig 3.1: High frequency stimulation (HFS) of Schaffer collaterals with a 200 Hz train for 1 s potentiates the amplitude of synaptically evoked transporter currents (STCs): A) Representative isolated STC traces before and at 2, 5, 15 and 30 min after the HFS. B) Current responses to a -5 mV step which preceded the STCs were used to control for changes in access resistance. C) Summary histogram shows that the STC amplitude increases gradually after the HFS and is significantly larger at 30 min after HFS. Numbers at the base of the histogram indicate number of cells (n). Data points in which access resistance changed ≥ 20% were discarded resulting in decreasing 'n' as the duration of the recording increased.

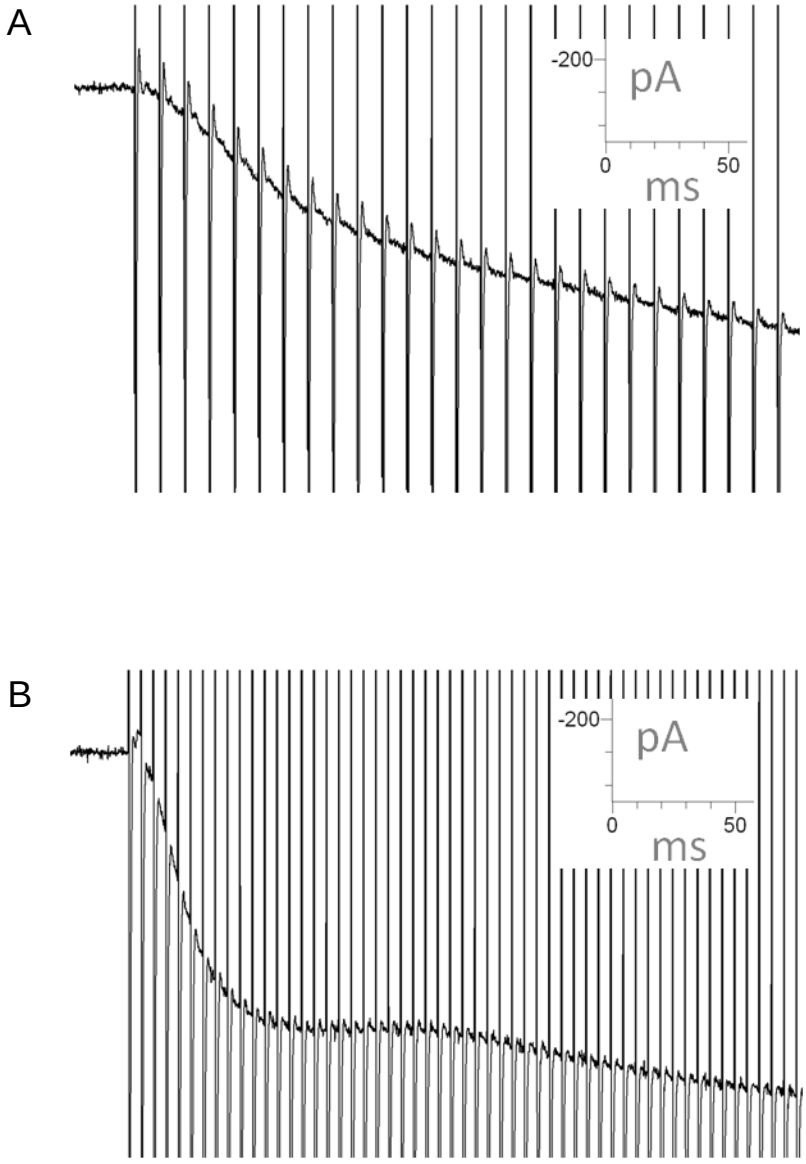

Fig. 3.2: STCs plateau after a 200 Hz stimulation: Examples of astrocytic inward currents evoked by a 100 Hz and 200 Hz train (75 µA, 200 µs pulses) are shown in A and B respectively. A 300 ms region from a 500 ms train has been clipped to better observe the difference between the two types of stimulation. Currents evoked by a 200 Hz train plateau at ~ 75 ms from the first stimulus whereas 100 Hz train evoked currents never reach a plateau.

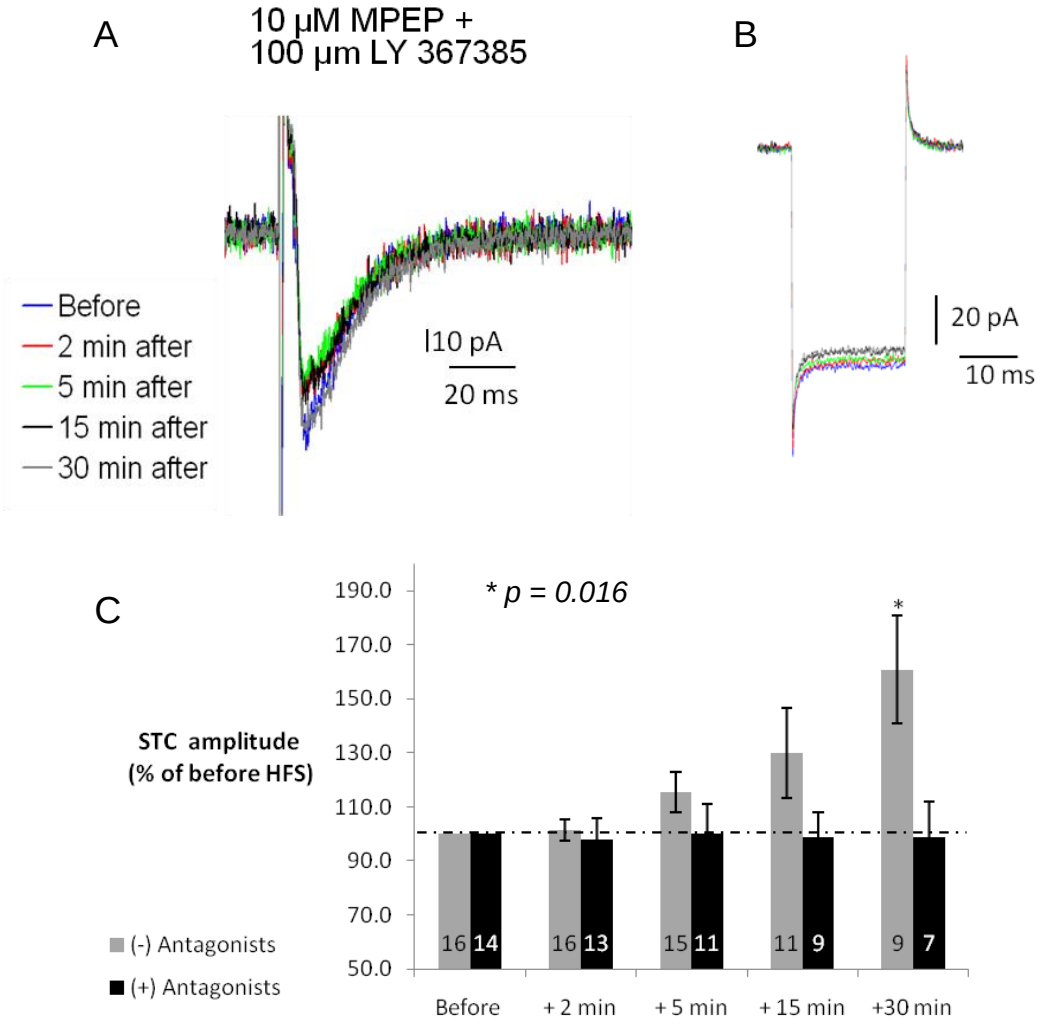

Fig. 3.3: Inhibiting group I mGluRs prevents HFS induced potentiation of STC amplitude: A) Representative isolated STC traces before and at 2, 5, 15 and 30 min after HFS in the presence of the group I mGluR antagonists, MPEP (10 µM) and LY 367385 (100 µM) in the bath show no increase in amplitude. B) Current responses to a -5 mV step which preceded the STCs were used to control for changes in access resistance. C) Summary histogram shows that the amplitude of the STCs did not increase after HFS in the presence of the group I mGluR antagonists (black bars). Number of cells is indicated in the base of the histograms. Gray bars representing amplitude of STCs from a different set of cells in the absence of antagonists (from Fig. 2.2) are included for comparison.

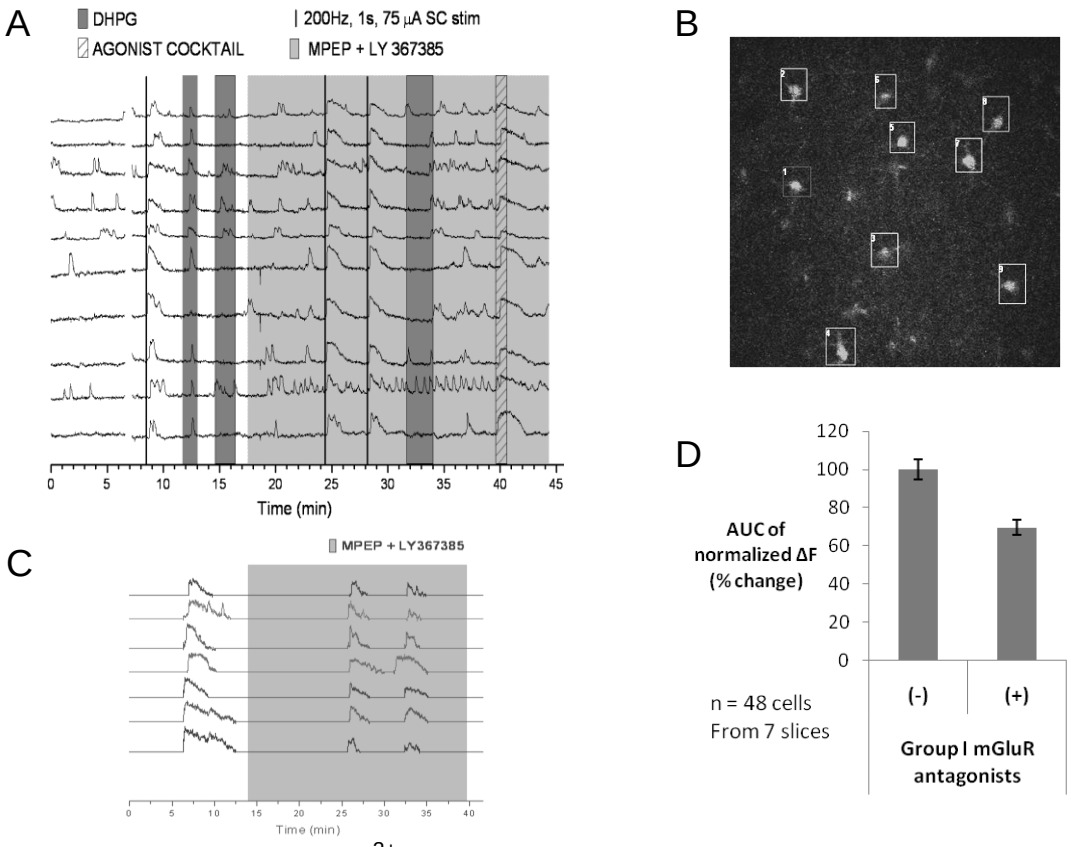

Fig 3.4: HFS induced [Ca^{2+}] elevations are only partially inhibited by group I mGluR antagonists: A) Normalized calcium responses of 10 different astrocytes bolus loaded with the calcium indicator dye, Oregon Green BAPTA -1 (OGB-1). In the presence of group I mGluR antagonists MPEP + LY 367385 (light gray), DHPG (50 μM) did not induce calcium elevations in astrocytes (dark gray), indicating the efficacy of the antagonists. However, HFS (black vertical lines) evoked calcium responses even in the presence of the antagonists. A cocktail of agonists for histamine receptors (Histamine, 10 μM) and muscarinic receptors (Carbachol, 10 μM) was capable of inducing astrocytic calcium responses in the presence of group I mGluR antagonists towards the end of the measurement period indicating viability of the astrocytic calcium responses. B) Confocal image of the bolus loaded slice from which the traces in A were obtained showing regions of interest boxes placed on the cell bodies of different astrocytes. C) Normalized HFS induced calcium responses of different astrocytes from another slice are isolated to better reveal the the reduction in the area under the curve (AUC) in the presence of group I mGluR antagonists. Baseline has been zeroed for clarity. D) AUC of normalized HFS induced calcium responses shows a partial (30%) but highly significant reduction ($p = 0.00006$) in the presence of group I mGluR antagonists.

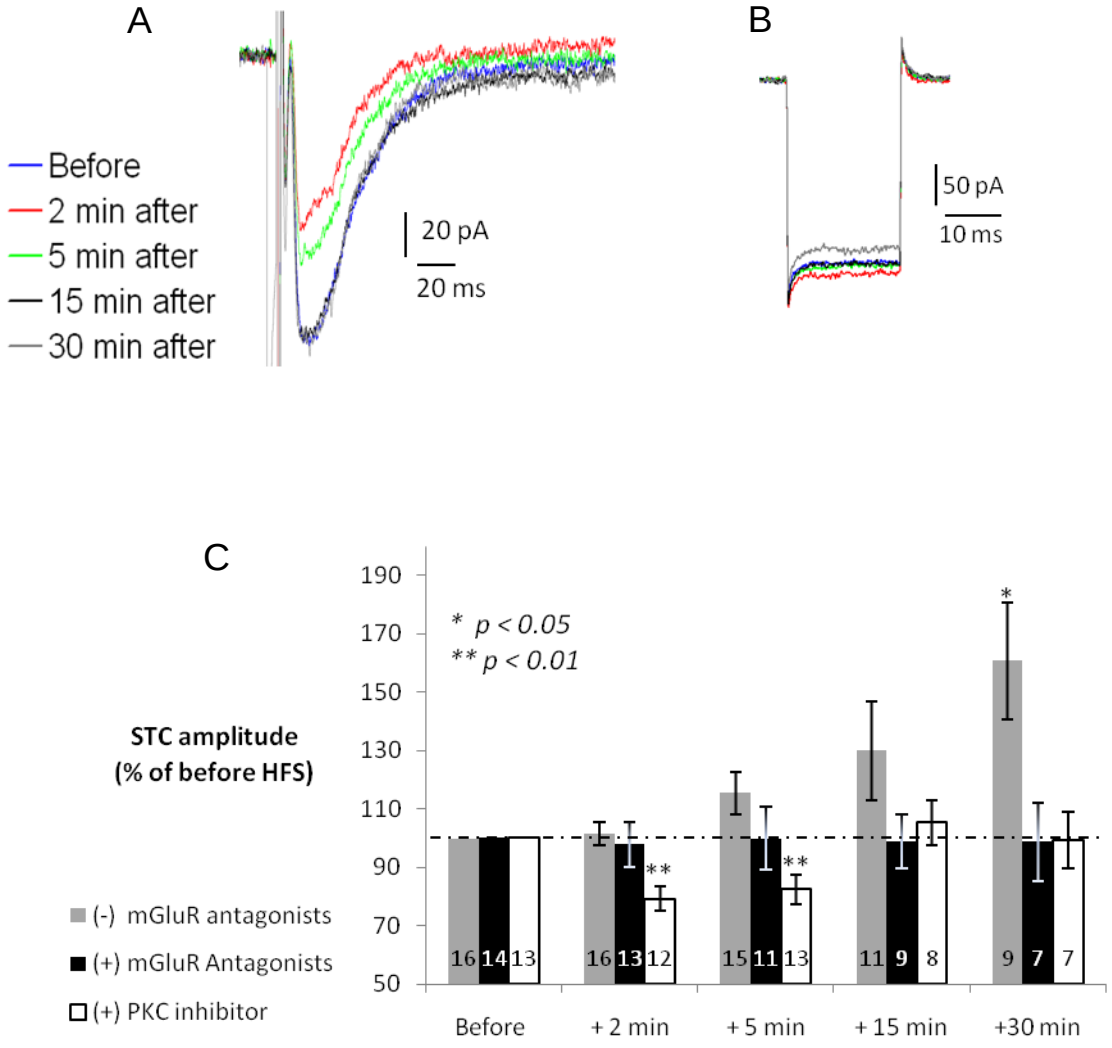

Fig 3.5: Inhibiting PKC prevents HFS induced potentiation of STC amplitude: A) Representative isolated STC traces before and at 2, 5, 15 and 30 min after HFS. B) Current responses to a -5 mV step which preceded the STCs were used to control for changes in access resistance. C) Summary histogram shows that the amplitude of the STCs did not increase after HFS in the presence of 10 µM of the PKC inhibitor, PKC 19-36 in the patch-pipette (white bars). In the presence of the PKC inhibitor, however, there was a significant decrease in the amplitude of STCs at 2 and 5 min after the HFS. Gray and black bars representing amplitude of STCs from a different set of cells in the absence and presence of group I mGluR antagonists, respectively, (from Figs. 2.2 & 2.3) are included for comparison (Refer to Fig. A1 in the Appendix for the distribution of amplitudes).

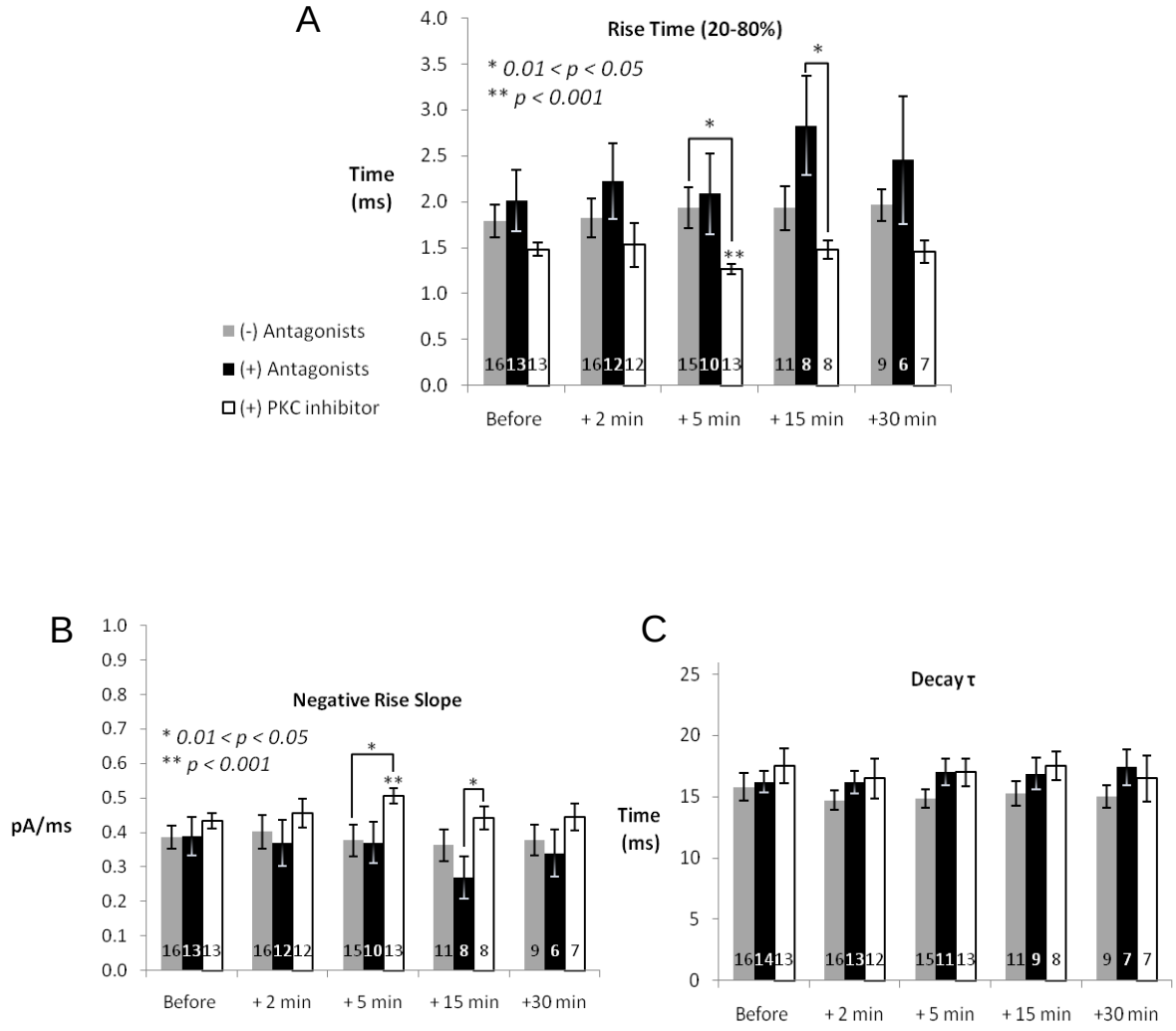

Fig 3.6: Effect of HFS on the kinetics of STCs: HFS did not lead to any significant difference in rise time (A), rise slope (B) and decay τ (C) of the STCs either in the absence (gray bars) or presence (black bars) of group I mGluR antagonists (MPEP 10 µm + LY 367385 100 µm). Inhibiting PKC intracellularly with PKC 19-36 (10 µM) led to a faster rise of STCs at 5 min after HFS (white bars) indicated by significantly faster 20-80% rise time (A) and steeper slope (B).

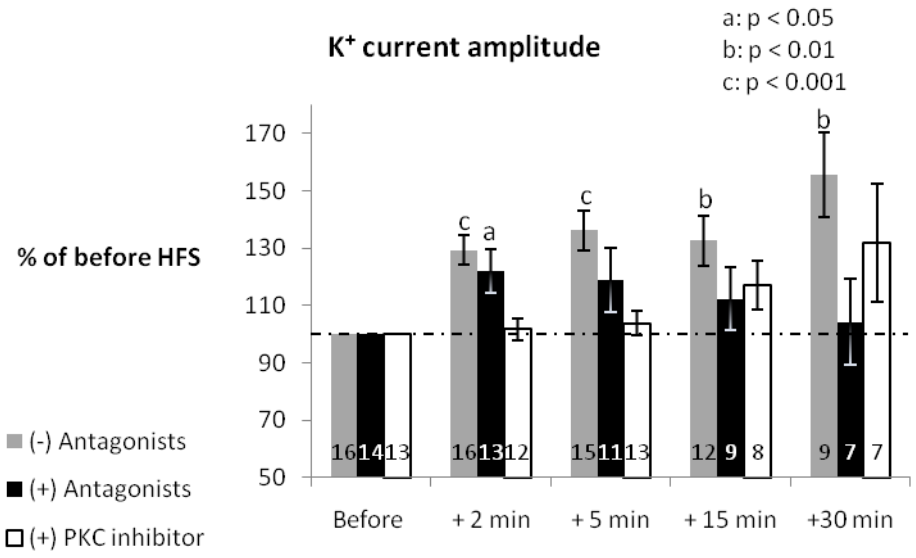

Fig. 3.7: HFS enhances K^+ current amplitude: The steady state amplitude of the K^+ current measured 75 ms after each test pulse shows significant increase after HFS at all measured time points (gray bars). In the presence of group I mGluR antagonists (black bars), a significant increase in K^+ current amplitude was observed only at 2 min after HFS. In the presence of PKC inhibitor, there was no significant change in K^+ current amplitude after HFS at any of the measured time points.

Chapter 4

Activation of Gq GPCRs Specifically Expressed by Astrocytes is Sufficient to Potentiate Glutamate Uptake

Abstract

The sufficiency of astrocytic Gq GPCR activation for potentiating glutamate uptake is tested in the experiments described in this Chapter. The recently developed MrgA1R transgenic mice was used to selectively activate astrocytic Gq GPCRs. Astrocytes in these mice selectively express the MrgA1R, an orphan Gq GPCR not expressed by any of the cells in the brain. This enabled us to study the effect of selective activation of astrocytic Gq GPCRs on glutamate uptake. Results suggest that activation of astrocytic MrgA1Rs is sufficient to potentiate glutamate uptake. Surprisingly, we also found an increase in the amplitude of the slow inward potassium current following astrocyte-specific Gq GPCR activation.

Introduction

The experiments detailed in Chapter 3 firmly suggest that activation of the Gq type of mGluRs (group I) is necessary for high frequency stimulation (HFS) induced potentiation of astrocytic glutamate uptake. However, a couple of concerns remain unanswered. As neurons also express group I mGluRs, there might be a contribution of neuronal mGluR activation, though unlikely, to the observed effect on astrocytic glutamate uptake. Enhanced glutamate release has been suggested to contribute at least in part to HFS induced LTP (Zakharenko et al., 2001) raising the possibility that astrocytic transporter currents are to some extent mirroring the increased glutamate release. These issues can be easily addressed if there is a ligand which will selectively activate astrocytic group I mGluRs. As astrocytes do not express unique mGluRs, we took advantage of the recently developed MrgA1R mice (Fiacco et al., 2007). These mice express a transgenic Gq GPCR, the MrgA1R, selectively in astrocytes. MrgA1R is not expressed by any other cells in the brain. MrgA1R is coupled to the same Gα subunit as group I mGluRs. This enabled us to wash in a peptide agonist of the MrgA1R, phe-met-arg-phe amide (FMRFa) to selectively activate astrocytic Gq signaling cascades thereby mimicking activation of group I mGluRs. We used these novel MrgA1R mice in the experiments described in this chapter to study the effect of astrocyte specific activation of Gq GPCRs on glutamate uptake. Results suggest that selective activation of astrocytic MrgA1Rs is sufficient to potentiate glutamate uptake. We also discovered a highly significant increase in

the slow inward K^+ current following MrgA1R activation, suggesting a synergistic regulatory mechanism for the uptake of both glutamate and potassium in astrocytes.

MrgA1R transgenic mice

MrgA1R stands for Mas-related-gene A1 Receptor. This is an orphan Gq coupled receptor expressed by nociceptive terminals in the spinal cord dorsal root ganglion (Dong et al., 2001). A mouse line expressing the MrgA1R selectively in astrocytes was recently generated using the inducible tet-off system (Fiacco et al., 2007). Following is a brief description of how this transgenic mouse was generated. MrgA1R was fused with the tetO minimal promoter in one mouse line. The tetracycline transactivator (ttA) was targeted to the astrocyte specific Glial Fibrillary Acidic Protein (GFAP) promoter in another mouse line. By crossing these two lines, MrgA1R can be transcribed off of the tetO promoter selectively in astrocytes. When the mice are bred in the absence of doxycycline, ttA binds to the tetO promoter leading to the expression of MrgA1R selectively in astrocytes (Fiacco et al., 2007). The founder mice for developing the MrgA1R colony in our lab were a gift from Dr. Ken McCarthy. Mice were routinely crossed with the wild-type C57BL/6J strain to avoid inbreeding. Mice were identified by toe-clipping at P7 and the toe-clip samples were sent to Transnetyx Inc. for genotyping before experiments. Only transgenic mice heterozygous for both the ttA allele (OT) and the MrgA1R allele (OM) were used for experiments.

Results

Experiment 4.1: Duration of FMRF wash-in to produce a calcium response

We used 10 µM FMRFa, a concentration which produces a plateauing calcium response (Fiacco et al., 2007), to activate the MrgA1R. A pilot experiment to measure the duration of wash in showed that a 90 s wash-in of FMRFa was sufficient (mean time to peak of 39.06 s; n = 9) to evoke a plateauing calcium elevation in astrocytes (Fig. 4.1) similar to that evoked by HFS.

Experiment 4.2: Effect of MrgA1R activation on astrocytic glutamate uptake

As this experiment was one of the first to be carried out in the project, we did not attempt to isolate the glutamate transporter component. Instead, we used paired pulse stimulation and measured the amplitude of the total current at the peak of the fast component in the facilitated current (current evoked by the second pulse). This current represents a complex of glutamate transport and potassium uptake. We measured the amplitude of this total evoked current before, during and at 2, 5, 15 and 30 min after a 90 s application of 10 µM FMRFa. We observed a significant increase in the amplitude of the total evoked current after FMRFa application both in MrgA1R and littermate control mice (Fig 4.2). However, the increase in evoked current amplitude was highly significant in MrgA1R group compared to littermate controls (*p* values: 0.000002, 0.00004, 0.0009 for MrgA1R; vs. 0.0002, 0.02, 0.015 for littermate controls at 2, 5 and 15

min respectively after FMRFa application). It was surprising that even the littermate controls which do not express the MrgA1R showed a significant increase in the evoked current amplitude after FMRFa application. This might be due to the activation of endogenous mGluRs by glutamate released during test pulses used to measure glutamate uptake. This hypothesis was tested in a subsequent experiment as described shortly. A contribution from a non-specific effect of FMRFa, although highly unlikely, cannot be excluded however. Compared to the HFS induced potentiation of STC amplitude which showed a significant increase at 30 min, the increase in the evoked current amplitude following FMRFa application occurred much earlier (a significant increase was observable as early as 45 s after FMRFa wash in). The temporal difference in the evoked current amplitude increase following HFS (described in Chapter 3) and FMRFa application along with the surprising amplitude increases following FMRFa application in littermate controls called for the next experiment testing the involvement of endogenous mGluR activation by glutamate released by test pulses.

Experiment 4.3: Effect of MrgA1R activation on astrocytic glutamate uptake in the presence of group I mGluR antagonists

The significant increase in total evoked current following FMRFa application in littermate controls which do not express MrgA1R suggested that the activation of mGluRs by glutamate released following test pulses might be contributing to the effect. In order to test this possibility, we compared STCs

evoked before and at 2, 5, 15 and 30 min after FMRFa application in the presence of group I mGluR antagonists (MPEP, 10 µM and LY367385, 100 µM). In this experiment, we employed the isolation protocol described in Chapter 2. This enabled us to isolate the effect on glutamate uptake from potassium uptake. We observed that even in the presence of group I mGluR antagonists, FMRFa application led to a small but significant increase in the STC amplitude in slices from MrgA1R$^+$ mice (11% increase at 5 min after FMRFa application, $p = 0.04$, $n = 8$) but not in littermate controls (Fig 4.3). This result suggests that activation of astrocytic Gq GPCRs is sufficient to potentiate glutamate uptake. However, the increase in amplitude of isolated STCs (11% at 5 min after FMRFa) is smaller than that observed in the absence of antagonists (35% at 5 min after FMRFa) in the earlier experiment, suggesting that activation of endogenous group I mGluRs is contributing to potentiation of the astrocyte currents. Another possible explanation for the relatively smaller increase in STC amplitude in the presence of antagonists is that the current measured in this experiment is the isolated STC and not the total current. This reason is also supported by the analyses described later which show a significant increase in K^+ current amplitude at later time points following FMRFa application.

Effect of MrgA1R activation in the presence of group I mGluR antagonists on STC kinetics

The use of the subtraction protocol for isolating the STC from the K^+ current in the experiment carried out in the presence of group I mGluR

antagonists allowed us to analyze the kinetic parameters. The rise time (20-80%) and decay τ of STCs in MrgA1R mice were 1.8 ms (n = 8; S.D. = 0.78) and 12.4 ms (n = 8; S.D. = 2.2) respectively (Fig. 4.4 A & C). The negative rise slope had a mean of 0.4 (n = 8; S.D. = 0.15) (Fig. 4.4B). The observed rise time and decay τ is comparable respectively to that reported in adult and P12-P14 slices (Diamond, 2005). As our experimental age group is P12-P18, we cannot directly correlate the age groups to those in the Diamond et al. study, which compared P12-P14 with adult mice. However, the similarity in rise time (20-80%) and decay τ suggests that the expression of the MrgA1R does not affect glutamate transporter activity. We observed a very small but significant difference in rise time and slope at 15 min after FMRFa application both in MrgA1R and littermate controls (Fig. 4.4 A&B). We also observed a very small but significant increase in decay τ at 2 min after FMRFa in MrgA1R but not in littermate control mice (Fig. 4.4 C). The magnitude of these differences in rise time, rise slope and decay τ as indicated by paired *t*-test are so small that they preclude interpretation.

Effect of MrgA1R activation on K^+ current amplitude following FMRFa

In contrast to the smaller increase in STC amplitude after FMRFa application in MrgA1R mice (11% at 5 min, $p = 0.03$), the amplitude of K^+ current showed a highly significant increase (30, 35 and 37 % increase at 2, 5 and 15 min after FMRFa application with respective *p* values of 0.00006, 0.00004 and 0.006). We also observed an increase in K^+ current amplitude in littermate

controls at 2 and 5 min after FMRFa application but with lower statistical significance compared to MrgA1R (respective *p* values at 2 and 5 min after FMRFa application: 0.0005 and 0.02 for littermates vs. 0.00006 and 0.00004 for MrgA1R mice). Even in the presence of group I mGluR antagonists, FMRFa application was followed by an increase in K^+ current amplitude in MrgA1R. Just as in the absence of antagonists, the K^+ current amplitude increase in MrgA1R mice showed greater significant difference compared to littermate controls (respective *p* values at 2, 5, 15 and 30 min after FMRFa application: 0.003, 0.001, 0.0001, 0.02 in MrgA1R vs. 0.02, 0.03, 0.06 and 0.2 in littermate controls). The greater significant increase in K^+ current amplitude observed in the absence of group I mGluR antagonists suggests a contribution from activation of endogenous mGluRs by glutamate released during test pulses for measuring astrocyte currents. The same may be the reason for the amplitude increases observed in littermates. However, as in the case of transporter currents, a non-specific effect of FMRFa cannot be ruled out, especially because the increase in K^+ current amplitude in littermates at 2 min after FMRFa application was highly significant ($p = 0.0005$).

Discussion

The results discussed above address a major concern with the experiments in the earlier Chapter regarding the sufficiency of astrocytic group I mGluR activation for potentiating glutamate uptake. The use of MrgA1R

transgenic mice let us to determine the effect of selective activation of astrocytic Gq GPCRs on glutamate uptake. Activation of these receptors has been shown earlier to evoke calcium responses similar to that evoked by group I mGluRs (Fiacco et al., 2007), validating the use of this tool to mimic group I mGluR activation. After MrgA1R activation, the total evoked astrocytic current increased in amplitude with greater statistical significance in MrgA1R mice compared to littermate controls. The significant increase in littermate controls raises two possibilities: (i) activation of mGluRs by the glutamate released during test stimuli in itself potentiates astrocytic K^+ and glutamate uptake over time; (ii) an unknown non-specific effect of FMRFa contributes to potentiation of total astrocyte currents. The second possibility seems less likely based on publications showing a lack of effect of FMRFa on several types of neuronal baseline and evoked synaptic activity (Agulhon et al.; Fiacco et al., 2007). The only suspected candidates to be modulated by FMRFa, the Acid Sensing Ion Channels (ASICs) are not activated at the pH ranges of this experiment. ASICs are activated by a sudden pH drop from 7.4 to 6.3, a steep change in pH not to be encountered in our buffered perfusion solution (Lingueglia et al., 2006). It is most likely that activation of endogenous mGluRs by the glutamate released by test pulses caused the increase in evoked current amplitude. It remains to be determined if there are as yet undiscovered receptors in the brain activated by FMRFa which may in part increase the astrocyte current amplitude observed in littermate controls. The issue of endogenous mGluR activation by glutamate released by

test pulses is addressed by measuring the effect of MrgA1R activation on glutamate uptake in the presence of group I mGluR antagonists. Even in the presence of group I mGluR antagonists, FMRFa application resulted in significant increase in glutamate uptake in MrgA1R mice but not in littermate controls suggesting that astrocyte specific activation of Gq GPCRs is sufficient to potentiate glutamate uptake. Compared to the HFS induced potentiation of STC amplitude which showed significant increase at 30 min, the increase in the evoked current amplitude following FMRFa application occurred much more rapidly (significant increase was observable at 45 s after FMRFa wash in). This left time shift in the effect on evoked current following FMRFa application compared to HFS is most likely due to the fact that the current measured in the FMRFa experiment is the total current (STC + K^+ current).

It is surprising to find that following FMRFa application, the amplitude of the K^+ current increased significantly. As this effect persisted in the presence of group I mGluR antagonists, this is most likely a direct effect of MrgA1R activation. The major inward rectifying potassium channel in astrocytes responsible for potassium uptake is Kir4.1 (Butt and Kalsi, 2006). While GPCR mediated modulation of potassium channels of the Kir3.0 family has been well documented (Stanfield et al., 2002), it is unknown if Kir4.1 undergoes similar modulation. Our results showing an increase in K^+ current following MrgA1R activation suggest that Kir4.1 can also be modulated by astrocytic GPCR activation. Given the synergism between glutamate and potassium uptake, it is

not surprising that both are modulated by astrocytic Gq GPCRs. The greater increase in K^+ uptake compared to glutamate uptake following MrgA1R activation raises a concern that our subtraction protocol to isolate transporter currents may be including some of the increased K^+ uptake as an increase in glutamate uptake. As uptake of both glutamate and potassium go hand-in-hand, it is difficult to study one in complete isolation from the other. A recent study using siRNA mediated knockdown of Kir4.1 in cultured cortical astrocytes reported that glutamate uptake is inhibited following Kir4.1 inhibition (Kucheryavykh et al., 2007). Conditional knockout of astrocytic Kir4.1 leads to early mortality disabling the use of the approach for studying glutamate transport in isolation from K^+ uptake (Djukic et al., 2007). We have recently established protocols for carrying out glutamate uncaging experiments using a circular bath which avoids run down of uncaging evoked currents encountered during the usual static bath procedure. This approach will enable us in the near future to evoke glutamate transporter currents in isolation from release of K^+ from neuronal synaptic terminals. Using this circular bath model, it will be possible to study the effect of astrocyte selective Gq GPCR activation on glutamate uptake in isolation from K^+ uptake and will also alleviate concerns regarding the increase in K^+ current masquerading as an increase in glutamate uptake.

Extracellular concentrations of potassium and glutamate are tightly regulated in the central nervous system by astrocytic uptake mechanisms (Kofuji and Newman, 2004; Tzingounis and Wadiche, 2007). Based on the results from

this dissertation study, we propose that astrocytic Gq GPCRs are intrinsic regulators of glutamate uptake. The results from K^+ current measurement are surprising and suggest synergistic modulation of both glutamate and potassium uptake by astrocytic Gq GPCRs. Future experiments are needed to further delineate the molecular mechanisms involved in the regulation of glutamate and potassium uptake.

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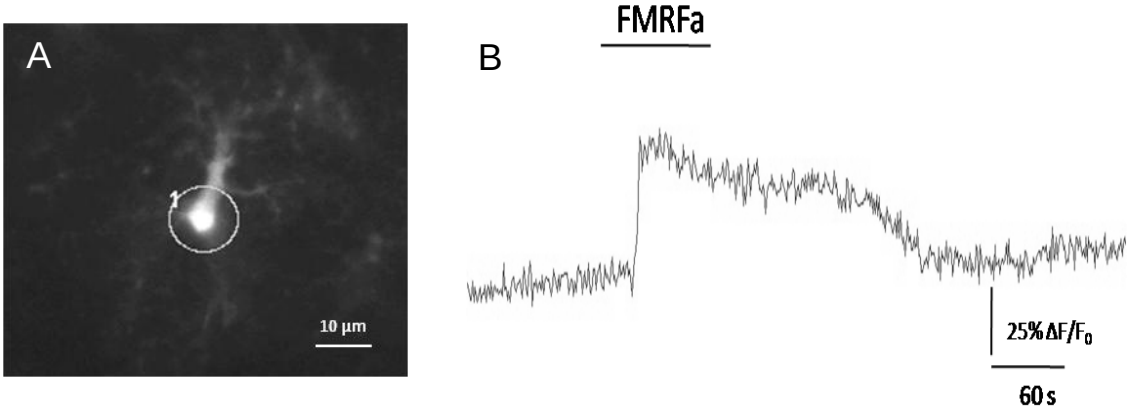

Fig. 4.1: FMRFa (10 µM) application for 90 s activates astrocytic MrgA1Rs: (A) A stratum radiatum astrocyte from MrgA1R mouse hippocampal slice patch-filled with 200 µM of the calcium indicator dye, Oregon Green BAPTA-1 (OGB-1). (B) Fluorescence trace obtained from the region of interest circled in (A) shows that a 90s application of FMRFa is adequate to evoke a plateauing calcium response, indicating expression and activation of functional MrgA1Rs in astrocytes.

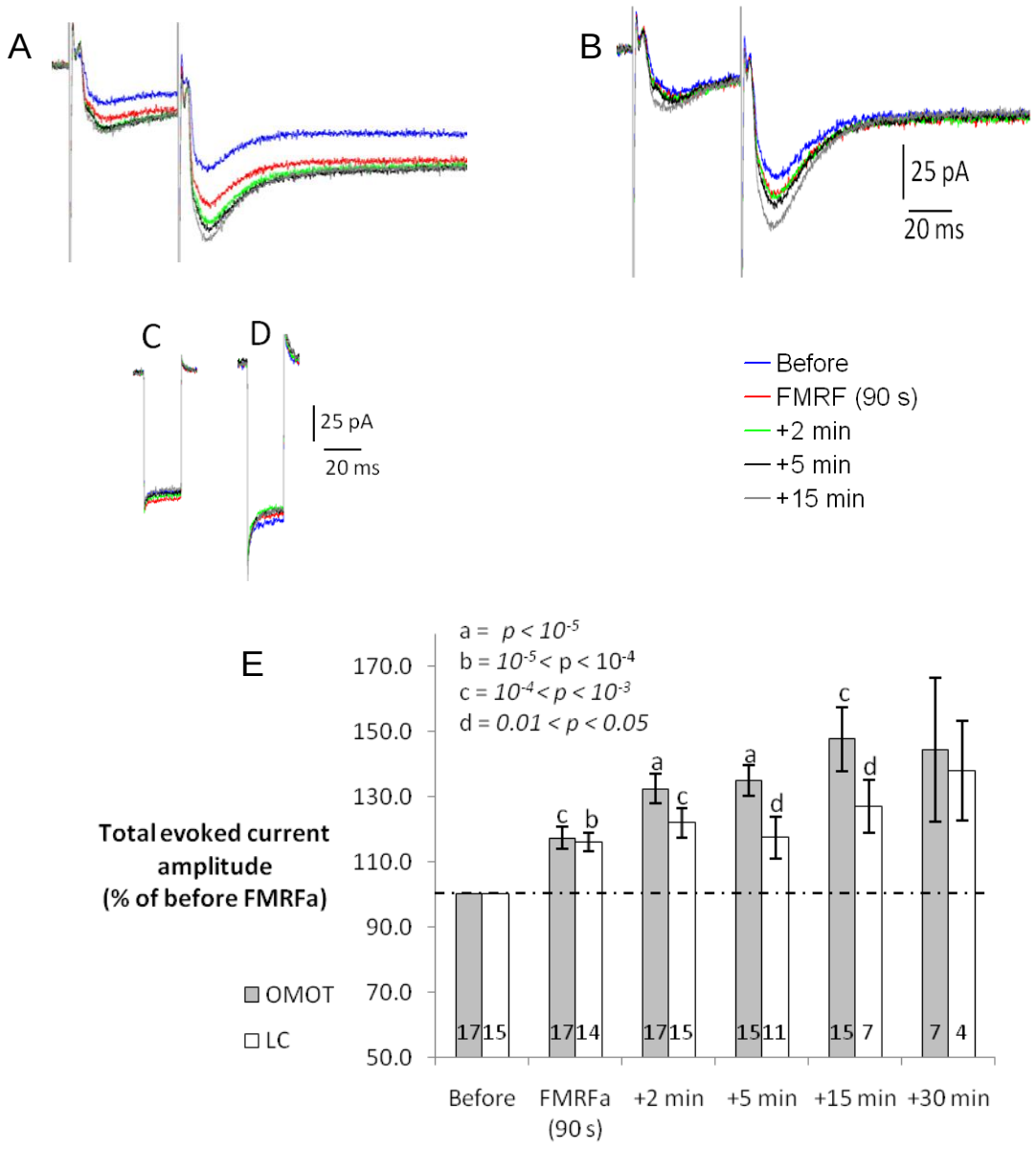

Fig. 4.2: Effect of MrgA1R activation on total evoked astrocytic current: Paired pulse stimuli (75 µA, 200µs, 20 Hz) to Schaffer collaterals were used to evoke astrocytic currents. Representative total evoked currents from MrgA1R (OMOT) mice (A) and littermate control (LC) mice (B). Currents in (C) and (D) are responses to a -5 mV step pulse preceding STCs in (A) and (B). A 20% limit was set for these responses to control for access changes. (E) Summary histograms show that the amplitude of the fast component of the facilitated current (current evoked by the second pulse) is significantly larger both in MrgA1R mice and littermate controls during and at 2, 5 and 15 min after MrgA1R activation by FMRFa application (10 µM, 90 s). However, the increase in MrgA1R mice was highly significant. (Refer to Fig. A2 in the Appendix for the amplitude distribution).

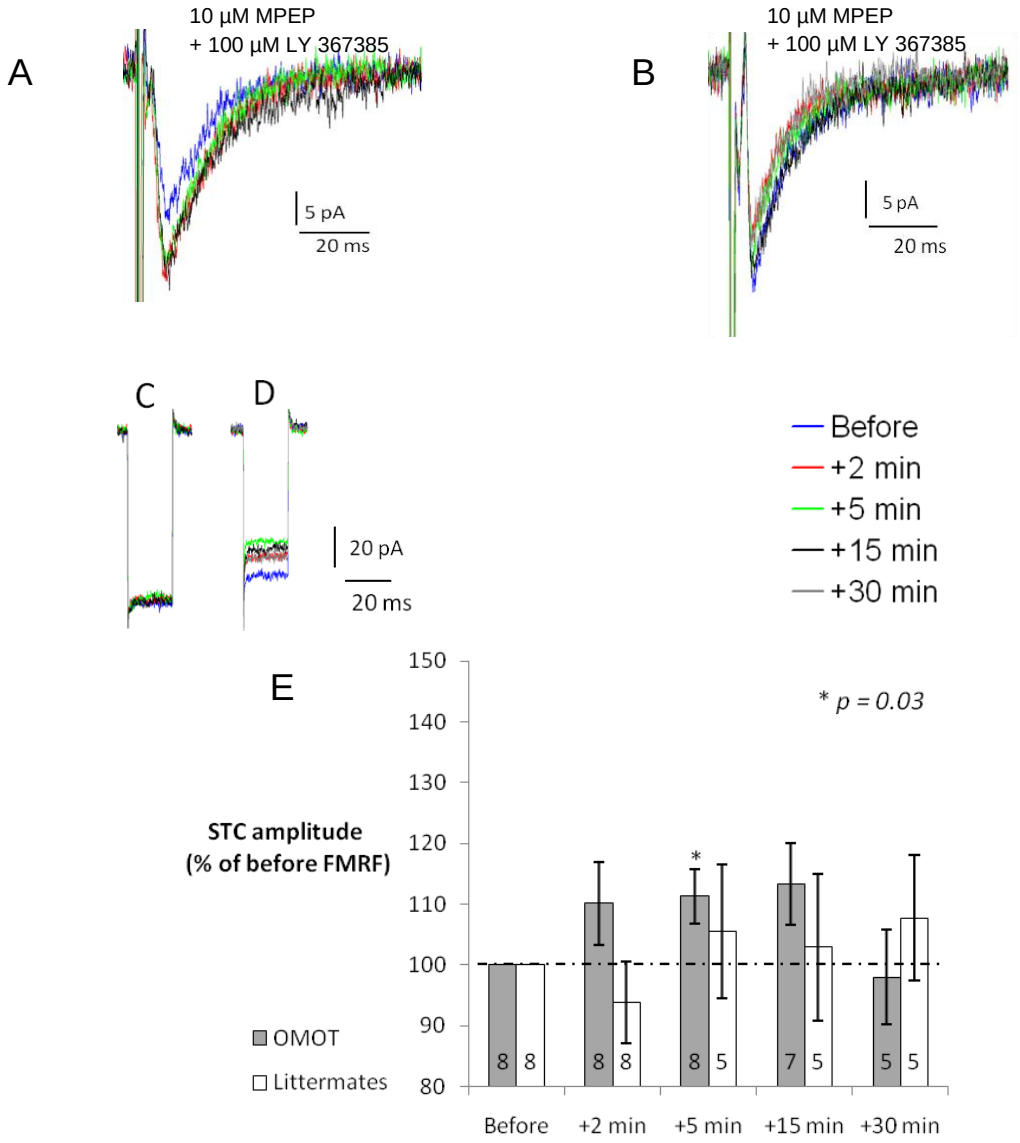

Fig. 4.3: Effect of MrgA1R activation on the amplitude of the isolated STCs in the presence of group I mGluR antagonists: STCs were isolated using the protocol described in Chapter 2. Representative STCs from MrgA1R (OMOT) (A) and littermate control mice (B) in the presence of group I mGluR antagonists (10 µM MPEP + 100 µM LY 367385) in the bath. Currents in (C) and (D) are responses to a -5 mV step preceding STCs in (A) and (B). A 20% limit was set for these responses to control for access changes. (E) Summary histogram shows that the amplitude of the STC is significantly larger in MrgA1R but not in littermate control mice at 5 min after MrgA1R activation using 90 s FMRFa (10 µM) application. These data suggest that selective activation of astrocytic Gq GPCRs is sufficient to potentiate astrocytic glutamate uptake. (Refer to Fig. A3 in the Appendix for the amplitude distribution).

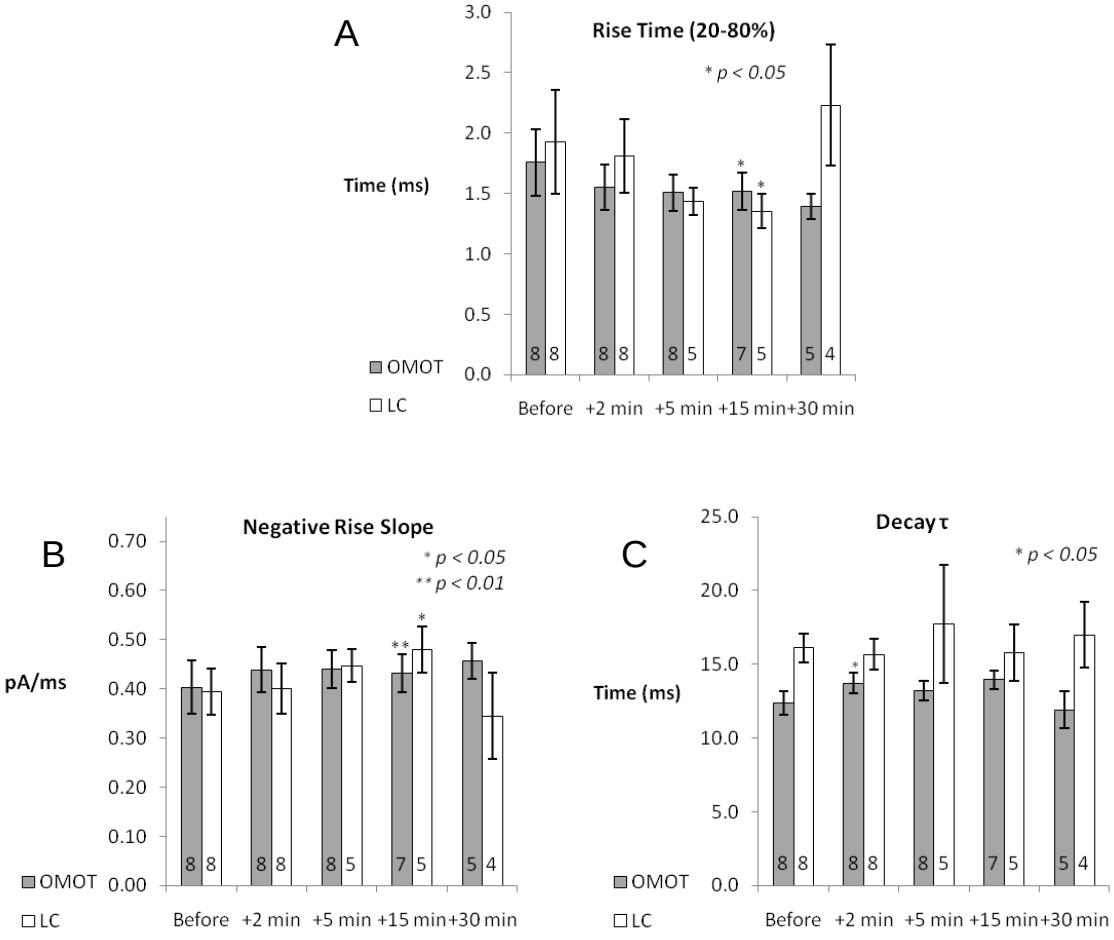

Fig 4.4: Effect of MrgA1R activation on the kinetics of STCs: The STCs isolated in the presence of group I mGluR antagonists, MPEP (10 µM) and LY 367385 (100 µM) in the bath, show a very small but significant decrease and increase, respectively, in rise time and slope at 15 min after FMRFa application (10 µM, 90 s) both in MrgA1R (OMOT) and littermate control (LC) mice. The effect of FMRFa application on decay τ is minor.

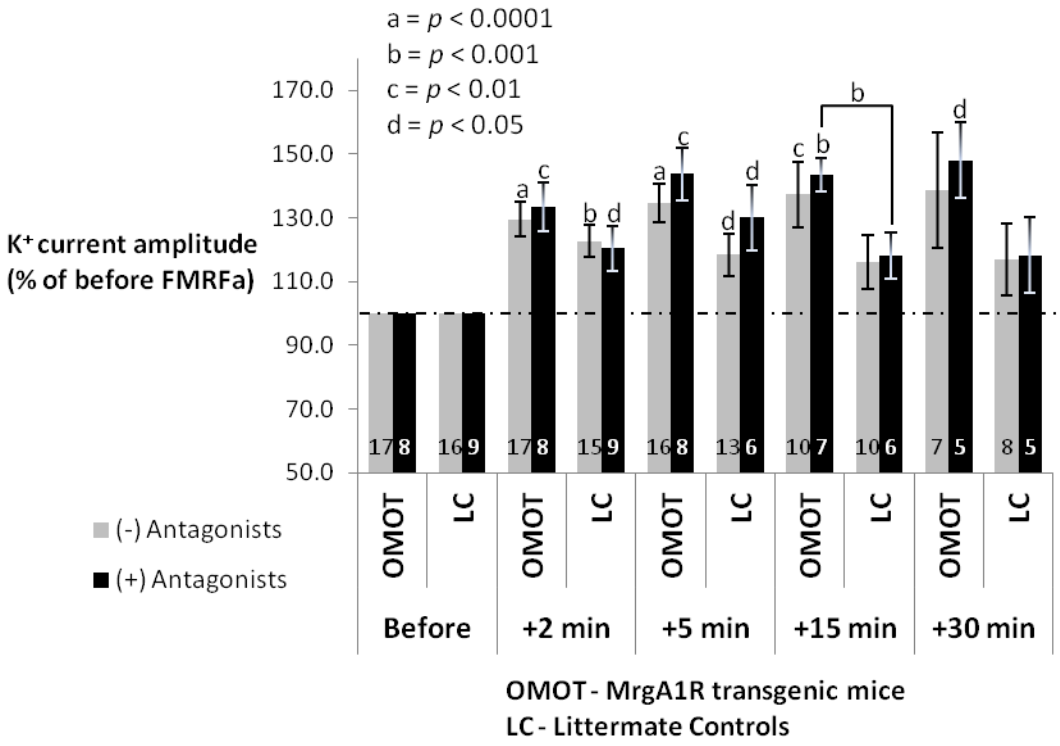

Fig. 4.5: MrgA1R activation potentiates K^+ current amplitude: The steady state amplitude of the K^+ current measured 100 ms after each test pulse shows significant increase after FMRFa application in the absence of group I mGluR antagonists both in MrgA1R and littermate control mice. However, the increase in amplitude was highly significant in MrgA1R mice compared to littermate controls. Even in the presence of group I mGluR antagonists, MrgA1R and littermate control mice showed significant increase in K^+ amplitude after FMRFa application. However, the increase in MrgA1R mice showed greater significant difference compared to littermate controls and lasted until the 30 min measurement suggesting an astrocytic Gq GPCR specific effect.

APPENDIX

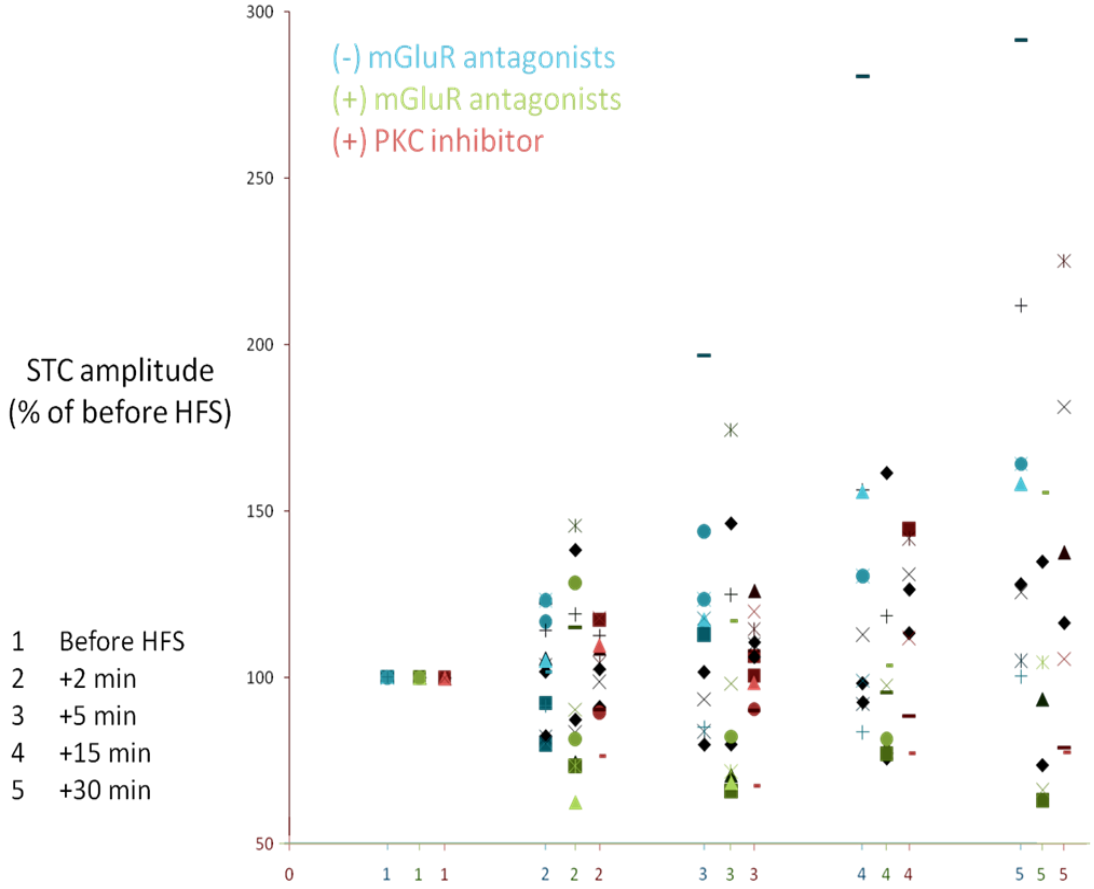

Fig. A1: Distribution of STC amplitudes after HFS under three different conditions: The distribution of STC amplitudes in the absence and presence of group I mGluR antagonists and in the presence of PKC inhibitor (PKC 19-36) is represented in this scatter plot. Each point in the plot represents STC amplitude from an individual cell. Color coded markers for the three different conditions represent different cells.

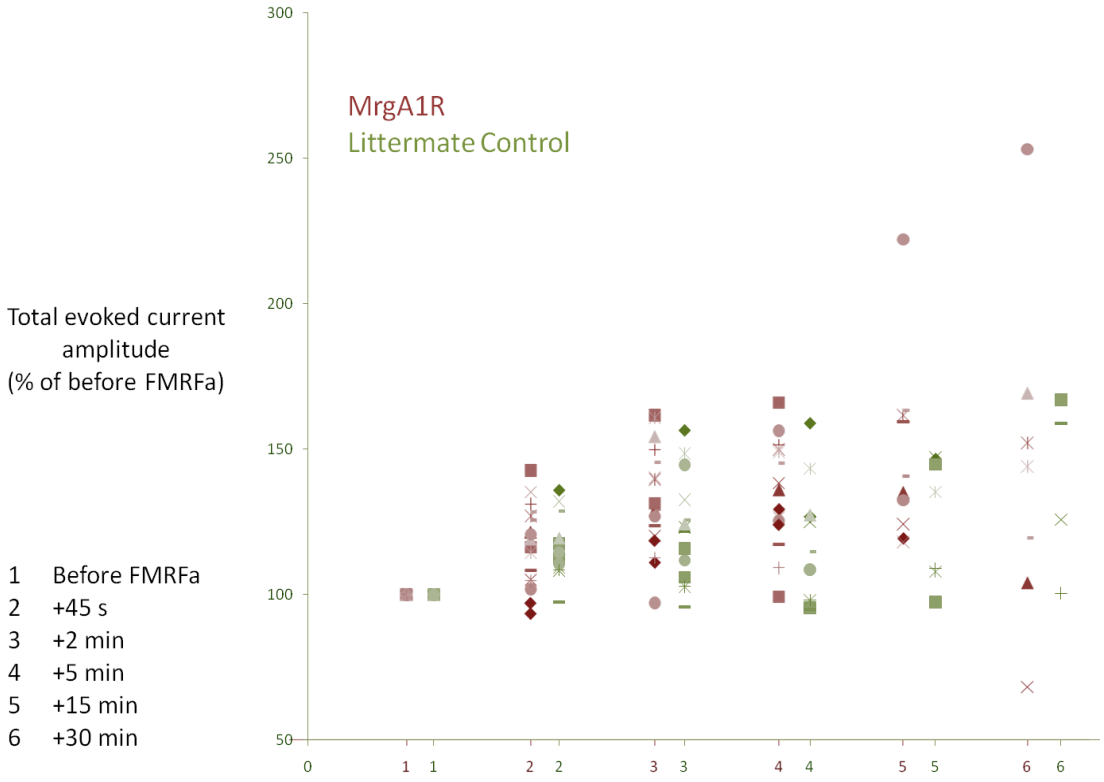

Fig. A2: Distribution of total evoked astrocytic current amplitudes from MrgA1R and littermate control mice after FMRFa application: The distribution of total evoked astrocytic current amplitudes from MrgA1R and littermate control mice following FMRFa application is represented in this scatter plot. Each point in the plot represents amplitude from an individual cell. Color coded markers for MrgA1R and littermate controls represent different cells.

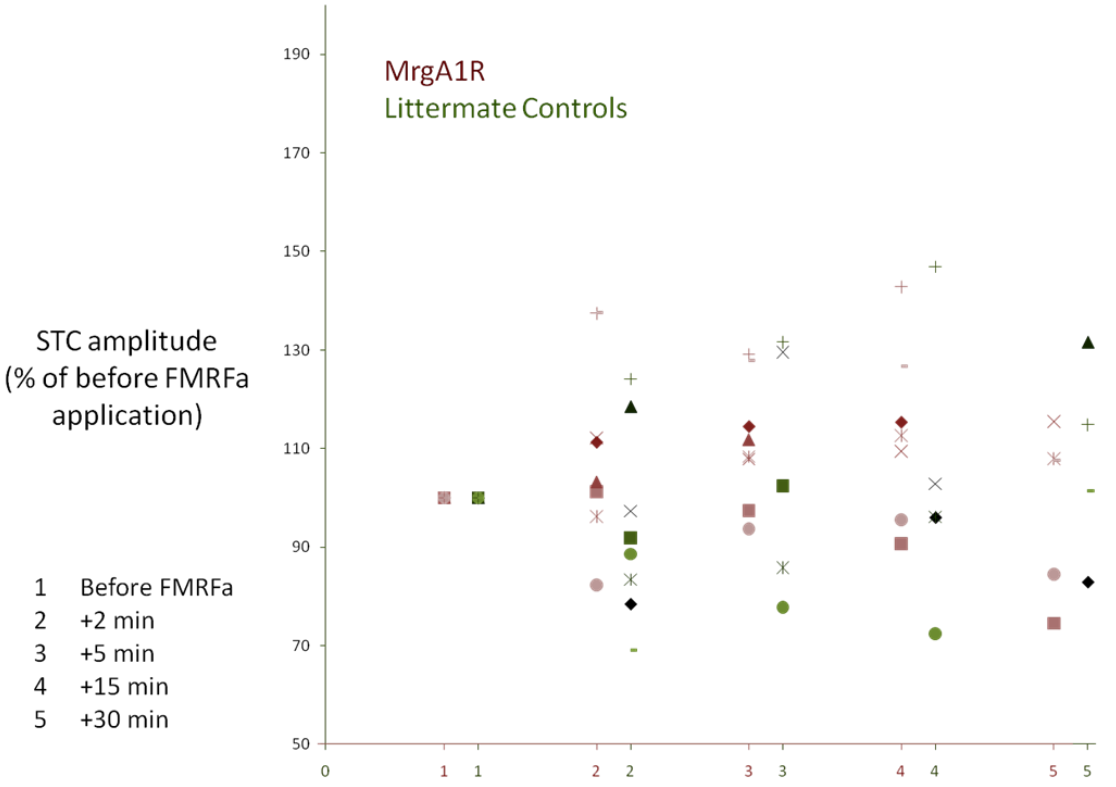

Fig. A3: Distribution of STC amplitudes from MrgA1R and littermate control mice after FMRFa application in the presence of group I mGluR antagonists: The distribution of STC amplitudes from MrgA1R and littermate control mice following FMRFa application in the presence of group I mGluR antagonists is represented in this scatter plot. Each point in the plot represents STC amplitude from an individual cell. Color coded markers for MrgA1R and littermate controls represent different cells.