Abstract

DOBRIN, SCOTT ERIC. N-acetylaspartylglutamate (NAAG) activates calcium responses of optic nerve glia. (Under the direction of Robert Grossfeld.)

At mammalian synapses, glia respond to neurotransmitters and can, in turn, modulate synaptic transmission. At non-synaptic regions, e.g. in optic nerve, glia also are sensitive to neurotransmitters, but the signaling agents and consequences for neural function are uncertain. ATP and glutamate produce similar responses in neonatal rat optic nerve as electrical stimulation and are, therefore, presumed to contribute to physiological or pathophysiological processes. Using calcium imaging, I tested whether calcium increases of optic nerve glia are activated by the glutamate-containing dipeptide N-acetylaspartylglutamate (NAAG). NAAG, as well as its hydrolyzing enzyme glutamate carboxypeptidase (GCP), is known to be present in rat optic nerve, and its stimulated release from optic nerve terminals has been reported. Studies have not been conducted, however, as to the possible role NAAG plays at non-synaptic regions of the optic nerve. NAAG is released from crayfish Medial Giant Axons upon electrical stimulation and causes a response in associated glia. It is presumed, therefore, to be a non-synaptic axon-glia signaling agent in that system. In vertebrates, NAAG has been speculated to influence myelination in peripheral nerve. Inhibition of GCP increases both the number of remyelinated axons and thickness of myelin after cryolesion in rat sciatic nerve. In the CNS, NAAG has been localized to optic nerve and may be an active non-synaptic axon-glia signaling agent there.

Optic nerves from rat pups (P5-9) were removed and desheathed. They were then incubated in the calcium-indicator dye Fluo-3 AM. Chemical agonists were bath-applied to the nerve and calcium transients were imaged using fluorescence microscopy. Bath-applied NAAG produced a dose-dependent calcium increase in the glia. A non-hydrolyzable form of

NAAG also increased glial calcium. When the nerve was preincubated in chemicals which increase the effectiveness of glutamate at glutamate receptors, a lower concentration of NAAG activated a response. Together, these results suggest that the NAAG response is likely to result from both intact NAAG and glutamate produced from it. Glutamate receptor agonists also were applied to determine what receptors might cause the responses seen. Agonists for AMPA receptors and metabotropic receptors caused a calcium increase. However, NMDA did not.

NAAG may be a physiological or pathophysiological non-synaptic signaling agent in the neonatal rat optic nerve, possibly, in part, by serving as a source of bioactive glutamate. Together with GCP, NAAG is likely involved in modulating myelination in the periphery, and possibly the CNS too.

N-ACETYLASPARTYLGLUTAMATE (NAAG) ACTIVATES CALCIUM RESPONSES OF OPTIC NERVE GLIA

by

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Biography

Scott Dobrin was born in Framingham, Massachusetts in 1981. He is the youngest of three children - with two older sisters. When he was five years old, he moved with his family to South Florida. He started training in Tang Soo Do shortly after coming to the area and became a black belt in 1991. After graduating from high school, he was admitted to the University of Florida, where he received his Bachelor of Science degree from the Department of Zoology in 2003. It was in Gainesville that he first became interested in scientific research. Scott came to Raleigh after graduating from UF and started at North Carolina State University the following January to pursue a Master of Science degree in the Department of Zoology. Throughout his life, Scott has worked as a cook and still enjoys his time fiddling in the kitchen – and eating the creations!

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Introduction

Axon-Glia Interactions

Glial cells make up half the volume of the mammalian brain and are ten-fold more numerous then neurons. However, their importance has historically been underestimated. In recent decades, bidirectional neuron-glia interactions have become evident. In both the peripheral and central nervous systems, chemical signals mediate communication between axons and glia. In the rat optic nerve, ATP and glutamate have been implicated as extra-synaptic signals between axons and glia (Kriegler and Chiu, 1993; James and Butt, 1999; Butt et al., 2004). It is the objective of this study to determine if NAAG, a glutamate containing dipeptide, also could be an axon-glia signal in neonatal rat optic nerve. NAAG (Williamson and Neale, 1988b), and its hydrolyzing enzyme glutamate carboxypeptidase (GCP; Sherman, Dobrin, Lubischer, and Grossfeld, unpublished data), have been localized to rat optic nerve and have been implicated in remyelination after peripheral nerve damage (Berger and Schwab, 1996; Shah et al., 1996; Yao et al., 1997) and a variety of neurological disorders (Slusher et al., 1999; Neale et al., 2005). In the study described in this thesis, it was shown that exogenously applied NAAG increased intracellular calcium in neonatal rat optic nerve glia, likely via activity of intact NAAG and glutamate produced from it.

The squid giant axon was the first preparation in which neuron-glia signaling was demonstrated. Villegas (1972) showed that Schwann-like glial cells had a biphasic electrical response to axon electrical activity. This was not due to increased extracellular K^+ during action potential generation, but rather was linked to acetylcholine release (Villegas, 1973). Since acetylcholine was found to be localized only to Schwann cell cytoplasm, this suggested that acetylcholine released from Schwann cells auto-activated the glia (Villegas, 1981).

Villegas (1978), initially, and Lieberman et al. (1989) subsequently, found that exogenously applied glutamate replicated the acetylcholine effect in squid and crayfish. That glutamate might be the axon-glia physiological signaling agent was suggested by the finding that glutamate receptor antagonists blocked the glial response to axon activity (Lieberman and Sanzenbacher, 1992). This led to the model that invertebrate giant axons release glutamate non-synaptically, that the glutamate acts on nearby Schwann cells to cause their release of acetylcholine, and that acetylcholine acts back on the glial cell membrane to cause a change in membrane potential (Fig. 1).

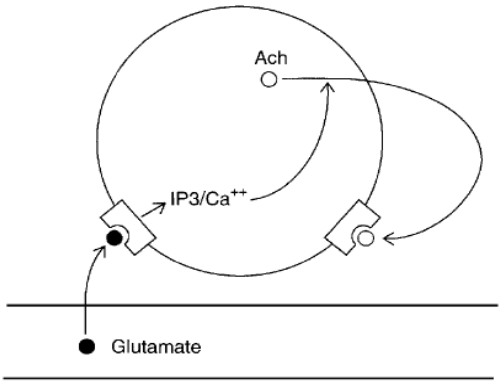

Figure 1. Model of axon-Schwann cell signaling in squid and crayfish giant nerve fibers. Glutamate is released from the axon and acts on neighboring Schwann cells. This causes a second messenger signal to increase release of acetylcholine from the Schwann cell. The Schwann cell membrane potential then changes. *From Chiu and Kriegler, 1994*

Weinreich and Hammerschlag (1975) first described non-synaptic release of glutamate in vertebrate central nervous tissue. After incubation of frog optic nerve in medium containing radioactive glutamate, nerves were electrically stimulated, causing a greater than 300% increase in radiolabeled glutamate release compared to the resting release. Not until the discovery that glial cells possess receptors for glutamate and other neurotransmitters did the prospect of synaptic and extra-synaptic neuron-glia signaling become evident. At the frog neuromuscular junction, Jahromi et al. (1992) showed that

motorneuron activation caused a Ca^{2+} increase in perisynaptic Schwann cells, and that the response was mediated by ATP, adenosine, and acetylcholine. Application of ATP elicited a dose-dependent uptake of radiolabeled Ca^{2+} in astrocyte cell culture (Neary et al., 1988). Kriegler and Chiu (1993) utilized Ca^{2+} imaging in rat optic nerve to show that glutamate had an affect on the resident glia. Together, these studies suggest that receptors for adenosine, ATP, and glutamate exist on various glial cells (Porter and McCarthy, 1997).

The neuromuscular junction is covered with non-myelinating Schwann cells, which can modulate synaptic activity. They have been shown to undergo a Ca^{2+} increase associated with neurotransmitter release from the presynaptic neuron. The activated Schwann cells then can depress neurotransmitter release from the motorneuron (Colomar and Robitaille, 2004). When a non-hydrolyzable form of GTP was injected into perisynaptic Schwann cells (thereby maintaining G-proteins in an active state), there was a decrease in the basal amplitude of end plate potentials (EPPs; Colomar and Robitaille, 2004). Miniature EPPs (which are caused by spontaneous neurotransmitter release) were not affected by the injection. When a non-hydrolyzable form of GDP was injected into the perisynaptic Schwann cells to maintain G-proteins in an inactive state, electrical stimulation of the presynaptic neuron did not show the synaptic depression which is typically induced by an appropriate stimulation. Together, these results indicate that glial cells, via G-proteins, mediate synaptic depression at the vertebrate skeletal neuromuscular junction.

Neuron-glia communication also is very important in the CNS. Astrocytic Ca^{2+} waves can modulate neuronal activity at great distances. Via Ca^{2+} waves, neurons can affect other neurons with which they do not make contact. In the hippocampus, for example, electrical stimulation of afferent neurons causes neurotransmitter release, which triggers

astrocytic waves of increased Ca^{2+} (Dani et al., 1992; Porter and McCarthy, 1996). Gap junctional coupling of glial cells and ATP released by astrocytes can promote propagation of the Ca^{2+} waves. Medium collected from astrocyte cell culture contained ATP during, but not before, Ca^{2+} wave propagation, and the waves were blocked by apyrase, which hydrolyzes ATP, or purinergic receptor antagonists (Guthrie et al., 1999). Parpura et al. (1994) showed that when single astrocytes in culture were experimentally stimulated with bradykinin (which causes Ca^{2+} to increase), glutamate was released, in turn increasing neuronal Ca^{2+}. Increased neuronal Ca^{2+} from bradykinin occurred only when the neurons were in co-culture with astrocytes. Together, these results indicate that neurotransmitter release that increases intracellular Ca^{2+} in astrocytes via axon-glia interaction is sufficient to increase neuronal Ca^{2+} via glial release of gliotransmitter. In this manner, bidirectional communication between neurons and glia exists at synapses.

The finding that ATP was co-released with GABA and acetylcholine from neurons and that all the major classes of glia had a broad range of purinergic receptors called attention to ATP as a possible major axon-glia signaling agent (Fields and Burnstock, 2006). ATP is an especially powerful chemical signal because it and each of the products of its hydrolysis can activate different types of receptors (Fields and Burnstock, 2006). Stevens and Fields (2000) showed that nerve activity inhibits proliferation and differentiation of glia in dorsal root ganglion, and its effect is mediated by ATP. Using time-lapse confocal microscopy to image Ca^{2+} changes in electrically stimulated neuron-Schwann cell co-cultures, Ca^{2+} was seen to increase in axons via voltage-sensitive Ca^{2+} channels immediately upon electrical stimulation but Ca^{2+} increased in Schwann cells only after a 15 – 150 sec delay. This is suggestive of a diffusible signal from the non-synaptic region of the axon to Schwann cells.

In a pure neuron culture, activity-dependent release of ATP was detected in the medium. To confirm that ATP was the diffusible signal causing the increase in Schwann cell Ca^{2+}, apyrase was applied to the co-culture. This blocked the Schwann cell response without affecting the increase in axonal Ca^{2+}. Using bromodeoxyuridine, which is incorporated in actively dividing cells and used as a marker for cell proliferation, it was shown that electrical stimulation of neurons and application of ATP both reduced the proliferation rate of the Schwann cells. However, in a later paper (Stevens et al., 2002), it was shown that ATP acting primarily through P2Y receptors, causes astrocytes to release leukemia inhibitory factor (LIF) which in turn promotes myelination, while adenosine, acting via A1 receptors, promotes differentiation and myelination in oligodendrocyte precursor cells (OPCs).

ATP also can promote propagation of astrocytic Ca^{2+} waves. Guthrie et al., (1999) examined "islands" of astroctyes, i.e. approximately 6 contiguous cells, in culture. They electrically stimulated one of the cells on an island and saw a Ca^{2+} wave pass throughout that cell's island and also increase Ca^{2+} in cells of non-contacting islands as far as 50 μm apart. This implied that a diffusible signal was involved. To prove this, they collected a portion of the medium during a Ca^{2+} wave and applied it to unstimulated astrocytes in another region of the culture. This caused a Ca^{2+} increase in those cells, whereas medium taken from around unstimulated astrocytes had no effect. Together, this proved that a substance was being released extracellularly from astrocytes that could excite other astrocytes. To determine what the chemical signal might be, the medium from around stimulated cells was collected and examined in two ways. First, it was applied to astrocytes in a controlled bioassay and its effect was compared to the effect of saline containing a known ATP concentration. Second, using a luciferase bioluminescence assay, the concentration of ATP in the medium from the

stimulated cells was independently determined. With both techniques, the ATP concentration was determined to be approximately 1 μM. Control samples were greater than 50-fold lower in ATP. Finally, it was found that locally applied ATP initiated a Ca^{2+} wave and that electrical stimulation in the presence of purinergic receptor blockers prevented the wave. This shows that astrocytic Ca^{2+} waves can be stimulated by released ATP. Pascual et al. (2005) used transgenic mice with altered SNARE proteins, which are thought to be involved in vesicular release of ATP, to examine their role in glial communication via ATP release. In animals with nonfunctioning SNARE proteins, extracellular ATP was reduced in response to electrical stimulation as compared to ATP release in animals with wild-type SNARE proteins. This suggests that astrocytes, in hippocampus, can release extracellular ATP in a vesicular-dependent manner and that ATP can then act as a gliotransmitter to stimulate a Ca^{2+} increase in other local cells.

ATP is not the only chemical likely to be active at synaptic and extra-synaptic sites – glutamate also is thought to be an axon-glia signaling agent. Glutamate has been implicated as a possible non-synaptic neuron-glia signal in the vertebrate nervous system based on its stimulated release (Weinreich and Hammerschlag, 1975) and activation of glial cell responses (Kriegler and Chiu, 1993; Kim et al., 1994). Glutamate release from astrocytes has been reported to involve several different mechanisms, including a mechanism similar to vesicular release from nerve terminals (Innocenti et al., 2000), a hemichannel-mediated release (Ye et al., 2003), release via transporter reversal (Szatkowksi et al., 1990), $P2X_7$ receptor-mediated release (Duan et al., 2003), and anion channel activation in response to cellular swelling (Basarsky et al., 1999). Hassinger et al. (1995) has shown that hippocampal neurons and astrocytes in co-culture communicate via released glutamate. Ca^{2+} waves were

elicited in the astrocytes by both mechanical and electrical stimulation of the astrocytes, leading to a subsequent increase in neuronal Ca^{2+}. It was found that tetrodotoxin, which blocks action potentials, did not decrease the neuronal Ca^{2+} increase. However, various glutamate receptor antagonists did. Both DNQX, which blocks non-NMDA ionotropic glutamate receptors, and AP5, an NMDA receptor blocker, reduced the neuronal response, but L-AP3, a metabotropic glutamate receptor blocker, did not. This implies that glutamate released from astrocytes in response to increased Ca^{2+} can cause hippocampal neurons to increase their Ca^{2+} and modulate neuronal activity either via activation of ionotropic receptors on hippocampal astrocytes (followed by the release of other substances from the astrocytes or through gap junctions) or on neurons.

Besides ATP and glutamate, optic nerve glia also respond to norepinephrine, histamine, serotonin, GABA, and acetylcholine (Kriegler and Chiu, 1993; Butt and Jennings, 1994; James and Butt, 1999). Of the compounds tested, exogenous glutamate or ATP were found to best mimic the change seen during electrical stimulation of the rat optic nerve (Kriegler and Chiu, 1993, James and Butt, 1999). It is currently hypothesized that both glutamate and ATP are involved in axon-glia signaling in the rat optic nerve (Fields and Burnstock, 2006).

Glutamate

Glutamate is generally considered to be the most abundant excitatory synaptic transmitter in vertebrate brain (Purves, et al., 2001). Although not as commonly studied at non-synaptic regions, glutamate does have activity there as well. It has been shown to depolarize glial cells at presumably physiological levels (Bowman and Kimelberg, 1984; Kriegler and Chiu, 1993; Montana et al., 2006) but also to produce excitotoxic cell death

when accumulating to excessive concentrations extracellularly (Lucas and Newhouse, 1957; Haas and Erdo, 1991; Yoshioka et al., 1995). Studies indicate that glial cells play an important role in taking up glutamate and thereby preventing glutamate excitotoxicity. Neuronal vulnerability to glutamate is increased in astrocyte-free cultures (Rosenberg and Aizenmen, 1989), and NMDA, which is more poorly transported than glutamate into glial cells from the extracellular space, is more potent than glutamate in cortical cell culture (Rosenberg et al., 1992). Knockdowns of glial glutamate transporters are associated with neurodegeneration typical of excitotoxicity and with progressive paralysis (Rothstein et al., 1996).

Glutamate has been shown to activate cultured CNS glial cells via both receptor activation and glutamate uptake (Bowman and Kimelberg, 1984; Oka et al., 1993; Melzer et al., 2005). Optic nerve glia possess a variety of glutamate receptors and transporters (Barcina and Matute, 1998; Alberdi et al., 2006; Micu et al., 2006; Cirone et al., 2002; Domercq et al., 1999). It is possible, therefore, that glutamate may activate rat optic nerve glia either via membrane receptors or cellular uptake.

Glutamate Receptors

Glutamate binds and activates two general types of membrane receptors: ionotropic and metabotropic. In order to understand the action of glutamate, one must consider the properties of these receptors.

Ionotropic Glutamate Receptors

Until the late 1980's, it was thought that glutamate only acted upon ionotropic receptors. Ionotropic receptors are those receptors which, when a ligand binds, change their conformation to allow ions to pass through a channel. Like all ligand-gated ion channels,

they are composed of a ligand-binding domain and an associated ion channel. There are three main types of ionotropic glutamate receptors, which were named from their specific agonists.

NMDA Receptor

N-methyl-D-aspartate (NMDA) receptors are highly permeable to Ca^{2+} and are permeable to Na^{+} and K^{+} to a lesser extent. These receptors are both ligand gated and voltage gated (Alford et al., 1989). NMDA receptors can be distinguished from other ionotropic glutamate receptors by their sensitivity to 2-amino-phosphonopentanoic acid (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). NMDA receptors are antagonized by AP5, but unaffected by CNQX; for the other ionotropic receptor types the opposite is true (McBain and Mayer, 1994). (+)-5-methyl-10,11-dihydro-5H- dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) also blocks NMDA receptor ion fluxes , by binding to a site located within the activated ion channel (Wong et al., 1986).

AMPA Receptor

The two other types of ionotropic glutamate receptors are often referred to as non-NMDA receptors due to their physiological similarities. One of these, the AMPA receptor, is preferentially activated by α–amino–3–hydroxy–5-methylisoxazole-4-propionate (AMPA). It is permeable to Na^{+} and K^{+} and to some extent Ca^{2+} (Bowie and Mayer, 1995). It undergoes desensitization, which effectively limits ion flow as a result of glutamate binding to the receptor for only a few milliseconds (Rosenmund and Mansour, 2002). Cyclothiazide (CTZ) blocks this effect. NBQX, which is an antagonist of non-NMDA receptors, does not distinguish between AMPA receptors and kainate receptors (the third ionotropic glutamate receptor type; Sheardown et al., 1990). Other classes of compounds do, however, selectively

antagonize AMPA receptors (Donovan et al., 1994; Menniti, 2000), such as 2,3-benzodiazepine derivatives, (R)-1-(4-aminophenyl)-4-methyl-7,8-methylenediazepine (GYKI 53784), for example, and quinazolinone derivatives, 3-(2-Chloro-phenyl)-2-[2-(6-diethylaminomethyl-pyridin-2-yl)-vinyl]-6-fluoro-3H-quinazolin-4-one (CP-465,022), for example.

Kainate Receptor

The other non-NMDA receptor type is the kainate receptor. As the name implies, the preferred agonist of this receptor type is kainic acid. Like AMPA receptors, kainate receptors are permeable to Na^+ and K^+ and to some extent Ca^{2+} (Bowie and Mayer, 1995). Kainate receptors also are highly susceptible to desensitization (Rosenmund and Mansour, 2002). CTZ does not block kainate receptor desensitization but concanavalin A does (Schiffer et al., 1997). In the past decade, selective kainate receptor antagonists also have been developed, such as methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroxyisoquinoline-3-carboxylate (LY382884) and (R,S)-3-(2-carboxybenzyl) willardine (UBP296) (Simmons et al., 1998; More et al., 2004).

Metabotropic Glutamate Receptors

In 1985, it was reported that glutamate stimulated inositol triphosphate (IP_3) formation in cultured striatal neurons through non- AMPA, -kainate, or -NMDA pathways (Sladeczek, 1985). Similar results were obtained in hippocampal slices (Nicoletti et al, 1986a), cultured astrocytes (Pearce et al, 1986), and cultured cerebellar granule cells (Nicoletti et al, 1986b). This led to the proposal that G-protein coupled glutamate receptors, i.e. “metabotropic” glutamate receptors (mGluRs), also existed. Since that time, eight such receptor subtypes have been identified. These have been subdivided into 3 groups based on

sequence homology, second messenger coupling, and pharmacology: group I (mGluR1 and 5 subtypes), group II (mGluR2 and 3 subtypes), and group III (mGluR4 and 6-8 subtypes) (Kew and Kemp, 2005).

The three mGluR groups can be distinguished pharmacologically. The most potent group I agonist is quisqualic acid. However, it also is an agonist of AMPA receptors, so it is not the first choice to distinguish between ionotropic and metabotropic receptors for many studies (Osborne and Larsen, 1998). Instead, 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) often is used (Kew and Kemp, 2005). A general group II agonist is 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC) (Kew and Kemp, 2005). To date, no known agonist activates all group III receptor subtypes. This is because mGluR7 is different from the other receptor subtypes in its low affinity for glutamate and other synthetic agonists (Ahmadian et al., 1999; Brabet et al., 1998). The agonist (S)-4-phosphono-2-aminobutyric acid (S-AP4) activates subtypes 4, 6, and 8, but not 7 (Kew and Kemp, 2005).

The three groups also can be selectively antagonized. A general group I mGluR antagonist is aminoindan-1, 5-dicarboxylic acid (AIDA) (Janáky et al., 2001). (2S)-2-ethylglutamate (EGLU) is a general group II antagonist (selective versus groups I or III) (Janáky et al., 2001). (R,S)-α-cyclopropyl-4-phosphonophenylglycine (CPPG) acts as general group III/II antagonist, with 20-fold selectivity for group III over group II (Kew and Kemp, 2005).

Table 1. Glutamate receptor agonists and antagonists.

Receptor	Agonist	Antagonist
NMDA	NMDA	AP5 MK-801
AMPA	AMPA Quisqualic acid	CNQX NBQX GYKI 53784 CP-465,022
Kainate	Kainic acid	LY382884 UBP296
Type I mGluR	Quisqualic acid ACPD	AIDA
Type II mGluR	APDC	EGLU CPPG
Type III mGluR	S-AP4*	CPPG

* S-AP4 does not activate the subtype 7 metabotropic glutamate receptor.

Inactivation of Glutamate Activity by Reuptake

Like most small molecule neurotransmitters except acetylcholine, glutamate in the extracellular space is removed by specific transporters. To date, five glutamate transporters have been identified. Two are expressed predominantly in glia [i.e. glial glutamate and aspartate transporter (GLAST) and glial glutamate transporter (GLT)], and three primarily in neurons [i.e. excitatory amino acid carrier 1 (EAAC1) and excitatory amino acid transporters 4 and 5 (EAAT4 and EAAT5)] (Meldrum, 2000).

In the CNS, EAATs keep extracellular glutamate levels low (Anderson and Swanson, 2000). This is thought to prevent excitotoxic cell death (Rothstein et al., 1996) as well as possibly play a role in synaptic plasticity (Turecek and Trussell, 2000). By controlling the amount of extracellular glutamate, the amount of presynaptic receptor activation is modulated, which affects transmitter release (Prieto et al., 1996; Maki et al., 1994). Tong and Jahr (1994) found that blockage of the glutamate transporters in rat hippocampal neurons attenuated postsynaptic excitation, and Brasnjo and Otis (2001) showed an mGluR-initiated

long-term depression at parallel fiber synapses via activation of postsynaptic mGluRs which may be linked to synaptic plasticity. Blocking glutamate uptake in the rat retina also caused neurodegeneration via activation of ionotropic glutamate receptors, presumably through excess extracellular glutamate accumulation leading to excitotoxicity (Izumi et al., 2002). Thus, the abundance and localization of both glutamate transporters and receptors are important for regulating glutamate's actions in biological systems.

Release of Glutamate

Glutamate is released from axon terminals in a Ca^{2+}-dependent manner involving N- and P/Q-type voltage-dependent Ca^{2+} channels (Birnbaumer et al., 1994). Typical presynaptic release of glutamate induces an excitatory postsynaptic potential, primarily due to the activation of AMPA receptors. Released glutamate can act back on the presynaptic cell to modulate the release (Meldrum, 2000).

Astrocytes also show a Ca^{2+}-dependent exocytotic release of glutamate in culture (Zhang et al., 2004). They express proteins, such as synaptobrevin II, cellubrevin, and syntaxin, which are known to form a neurotransmitter vesicle fusion complex in synaptic terminals (Parpura et al., 1995; Hepp et al., 1999). Furthermore, injection of Botulinum toxin B, which cleaves synaptobrevin, reduces the Ca^{2+}-dependent glutamate release (Jeftinija et al., 1999; Araque et al., 2000). This evidence suggests that glutamate can be released from astrocytes via a vesicular mechanism.

In addition to the more traditional vesicular release of glutamate from nerve terminals, glutamate also can be released non-vesicularly from non-synaptic regions of nerve fibers during electrical stimulation, presumably by the reversal of a glutamate reuptake transporter (Wheeler et al.1966). In conditions of high extracellular potassium and high

intracellular sodium, a Ca^{2+} -independent release of glutamate occurs (Brew and Attwell, 1987). Szatkowski (1990) showed that increasing extracellular potassium around glial cells produced an outward current, indicative of reversed glutamate transport. Evoked increases in extracellular glutamate due to ischemic rundown of ion gradients occur in cultured astrocytes (Longuemare and Swanson, 1995) and hippocampal slices (Phillis et al., 1998) through reversal of Na^{+}-dependent glutamate transporters. Glutamate released from Müller glia by reversal of glutamate transporters can activate glutamate receptors in adjacent salamander Purkinje neurons (Billups and Attwell, 1996). In culture, hippocampal astrocytes also have been shown to release glutamate via a hemichannel-mediated mechanism (Ye et al., 2003). Hemichannels, half of a gap junction, are closed in the presence of extracellular multivalent cations. When the cultured astrocytes were bathed in divalent cation-free saline, extracellular glutamate was increased. The increase was not seen when a variety of gap junction blockers were present. While this does not prove glutamate is released through the hemichannel pore, that is the most likely mechanism of release. Astrocytes in culture also release glutamate via activation of $P2X_7$ receptors (Duan et al., 2003). After being preloaded with radiolabeled glutamate, extracellular levels of the labeled amino acid were increased upon application of ATP to the medium. This could be blocked by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), a $P2X_7$ receptor antagonist, or oxidized ATP. Another non-vesicular mechanism of glutamate release has been shown to occur via anion channels in response to cell swelling (Basarsky et al., 1999). In hippocampal brain slices, spreading depression was induced by application of ouabain, resulting in cellular swelling. When NMDA receptor antagonists were applied, swelling was reduced, implying that glutamate was involved. In an effort to determine how glutamate was released, a blocker of volume-selective organic ion

channels was applied to the slice and a reduction in cellular swelling was seen. To confirm that blocking these channels affected glutamate release and was not working via another mechanism, glutamate levels in the superfusate of the slice were determined via HPLC. A reduction in released glutamate levels was found.

NAAG

Discovered in horse brain (Curatelo et al., 1965) during a search for neurotransmitter candidates, N-acetylaspartylglutamate (NAAG) is considered by some to be the most abundant peptide neurotransmitter or neuromodulator in the mammalian central nervous system (Coyle 1997; Neale et al., 2000). The dipeptide has been detected in concentrations as high as 4 mM in rat central neurons (Fuhrman et al., 1994). In the mid-1980's, antibodies were developed which allowed the distribution of NAAG immunoreactivity to be determined in the amphibian, rat, cat, primate, and human nervous systems (Moffet et al., 1989; Blakely et al., 1988; Cangro et al., 1987; Joëls et al., 1987; Kowalski et al., 1987; Williamson and Neale, 1988b; Tieman et al., 1987; Tieman et al., 1991; Tieman et al., 1988). NAAG has been shown to be widespread in brain regions, such as in ascending and descending spinal axons, spinal motorneurons, retinal ganglion cells, the nigrostriatal pathway, some cerebellar afferent neurons, neurons of the deep cerebellar nuclei, large spinal sensory neurons, and cortical interneurons (Fuhrman et al., 1994; Moffett et al.,1993; Cunningham and Sawchenko, 1990; Lavollée et al., 1987; Kowalski et al., 1987; Anderson et al., 1987; Tieman et al., 1987; Tsai et al., 1993b; Moffett et al., 1994). In addition to being co-localized in glutamatergic neurons, NAAG has been identified in neurons that use a variety of other transmitters, such as GABA, serotonin, norepinephrine, dopamine, or acetylcholine (Forloni et al., 1987; Gupta et al., 1992; Brovia et al., 1996). However, its function has not

been as well defined as that of other putative neurotransmitters.

NAAG is released from brain slices by a calcium-dependent, depolarization-induced process (Williamson and Neale, 1988a; Zollinger et al., 1988). In addition, ultrastructural studies showed its localization in synaptic vesicles. Williamson and Neale's (1988a) observations suggested the possibility that NAAG is a neurotransmitter. In order to conclude this, though, NAAG must also be able to alter the activity of a postsynaptic neuron at physiological concentrations.

NAAG has been shown to be an agonist of type II metabotropic glutamate receptors, showing preference for subtype 3 (Wroblewska et al., 1993; Bischofberger and Schild, 1996). NAAG also can activate NMDA receptors, but not AMPA or kainate receptors (Valivullah et al., 1994). In voltage clamp studies, NAAG acting on NMDA receptors caused an inward current, although its action was far less potent then either L-glutamate or NMDA (Westbrook et al., 1986). Sekiguchi et al. (1989) found that NAAG inhibited glutamate activation of NMDA receptors. In addition, [^{3}H]-norepinephrine release via glutamate activation of NMDA receptors was inhibited by NAAG application (Puttfarcken et al., 1993). For these reasons, it is thought that NAAG can act as a weak NMDA agonist pharmacologically, but physiologically it is likely to be an inhibitor of glutamate activation of NMDA receptors.

There is strong evidence implicating NAAG as an axon-glia signaling agent in the crayfish Medial Giant Nerve Fiber (Urazaev et al., 2001a, 2005; Gafurov et al., 2001). When incubated with radiolabeled glutamate or glutamine, these axons synthesize and release radioactive NAAG upon electrical stimulation (Urazaev et al., 2001). NAAG can activate a cascade of glial second messenger signals resulting in the hyperpolarization of the glia (Gafurov et al., 2001). Interestingly, NAAG stimulates NAAG peptidase activity in those

glia (Urazaev et al., 2005). Thus, NAAG activity on glial receptors can regulate NAAG's own hydrolysis to terminate its biological activity.

NAAG Peptidase

To study the reuptake of NAAG, Blakely et al. (1986) used N-acetylaspartyl-[^{3}H]-glutamate with a synaptosomal preparation. Instead of finding the radiolabeled NAAG accumulating, as was expected, they found that [^{3}H]-glutamate accumulated. This was blocked by quisqualate, which is an AMPA receptor antagonist - not a glutamate uptake inhibitor. Together, these experiments led to the conclusion that a peptidase existed which hydrolyzed NAAG to form glutamate and N-acetylaspartate (NAA) since NAAG was being cleaved to form glutamate and that formation was blocked in the presence of quisqualate (Fig. 2;. Robinson et al., 1986; Serval et al., 1990). This enzyme was first named N-acetylated α-linked acidic dipeptidase (NAALADase). Later it was found that the enzyme more generally cleaved substrates at gamma glutamyl peptide bonds and thus it was renamed glutamate carboxypeptidase (GCP) II (Robinson et al., 1987; Pinto et al., 1996). More recently, a second GCP isozyme with similar properties was discovered and named GCP-III (Bzdega et al., 2005). NAA, in addition to being a substrate for myelin formation (Gay et al., 1991), has been shown to activate both NMDA receptors and metabotropic glutamate receptors (Rubin et al., 1995; Yan et al., 2003).

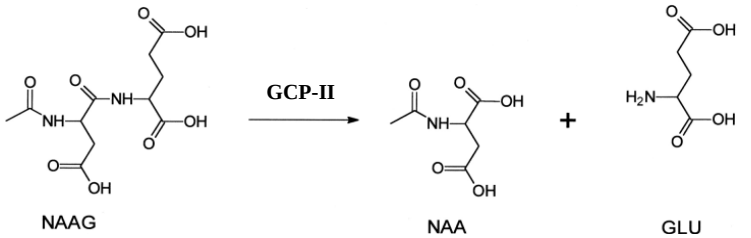

Figure 2. NAAG hydrolysis. The dipeptide NAAG is hydrolyzed in the extracellular space by NAAG peptidase, a.k.a. glutamate carboxypeptidase II (GCP-II), resulting in formation of glutamate and NAA. In this way, NAAG may serve as a source of glutamate.
Adapted from Neale et al., 2000

GCP-II is a glial cell ectoenzyme – a membrane-bound enzyme situated such that its active site faces the extracellular environment (Cassidy and Neale, 1993b; Berger et al., 1995). Using enzyme activity measurements and immunohistochemistry, the enzyme was found to be widespread throughout the nervous system, corresponding reasonably well with regional concentrations of NAAG (Slusher et al., 1990; Slusher et al., 1992; Blakely et al., 1988). Further studies have shown GCP-II to be present on astrocytes and immature Schwann cells (Cassidy and Neale, 1993b; Berger and Schwab, 1996). Interestingly, GCP-II activity is modulated in development. Enzyme activity is maximal at 7 – 15 postnatal days in rat optic nerve (Sherman, Dobrin, Lubischer, and Grossfeld, unpublished data) and at 14 – 15 postnatal days in rat sciatic nerve (Berger and Schwab, 1996). This is about the time of onset of myelination in both nerves, suggesting a possible role for NAAG and GCP-II in regulating myelin formation (Foster et al., 1982; Berger and Schwab, 1996).

Rodent Optic Nerve

The rodent optic nerve is a well-established model tissue in which to study axon-glia interactions (Bolton and Butt, 2005; Butt et al., 2004; Butt and Ransom, 1993; Kriegler and Chiu, 1993). It is easily dissected, contains no neuronal cell bodies or synapses, and, in the adult, has several glial cell types, namely astrocytes, oligodendrocytes, NG2-glia (synantocytes), and microglia (Butt et al., 2004). Using electron microscopy, immunohistochemistry, electrophysiology, and intracellular dye injections, glia of the rat optic nerve have been characterized (Ransom et al., 1991; Butt et al., 1994; Butt and Ransom, 1989; Butt et al., 1999). AMPA receptors, kainate receptors, NMDA receptors, and all three types of metabotropic glutamate receptors have been identified on rat optic nerve glia (Barcina and Matute, 1998; Alberdi et al., 2006; Micu et al., 2006; Cirone et al., 2002).

The glutamate transporters EAAC1, GLAST and GLT-1 also have been localized to optic nerve glia (Domercq et al., 1999).

Types of glia

Astrocytes are abundant in the optic nerve and important for its function. They contact axons at Nodes of Ranvier and are involved in a variety of activities, such as maintaining K^+ homeostasis and metabolism, as well as axon-glia signaling (Butt and Ransom, 1993; Ransom and Orkand; 1996). They have receptors for various neurotransmitters, including ATP, glutamate, and GABA (James and Butt, 2001; Butt and Jennings, 1994; Jeffrey et al., 1996). Neighboring astrocytes are connected via gap junctions, which allow electrical communication and the intercellular exchange of small chemicals (Massa and Mugnaini, 1985; Lee et al., 1994).

Oligodendrocytes, which are responsible for myelination of CNS axons, are first observed in the rat optic nerve 2 days after birth and then differentiate during the first 2 weeks of life (Butt and Ransom, 1993; Durand and Raff, 2000). It has been suggested, based on various characteristics of myelin development and growth, such as proper formation of Nodes of Ranvier while myelin segment length and thickness varies, that oligodendrocytes do not myelinate axons in a random manner. This suggests that intercellular communication, among the glial cells and/or between them and neighboring axons, is occurring.

Synantocytes have been considered to be a sub-class of oligodendrocyte precursor cells (OPCS). They are post-mitotic cells that are morphologically similar to astrocytes but are physiologically distinct from them. They do not express the stereotypical astrocyte marker glial fibrillary acidic protein (GFAP), nor do they express glutamine synthetase (Butt et al., 2005). They are distributed fairly uniformly throughout the nerve, in the same regions

as myelinating cells (Butt et al., 2004). These cells express various ionotropic glutamate receptors, which could be used to monitor axon activity via a response to released glutamate (Fulton et al., 1992; Yuan et al., 1998). They are thought to participate actively in CNS tissue responses to injury, e.g. reactive gliosis and remyelination of axons.

Microglia are the resident macrophages of the brain and optic nerve (Visentin et al., 1999). In their resting state, they appear to monitor a defined volume of tissue for signs of injury (Färber and Kettenmann, 2005). Upon trauma, they are activated and migrate to the site of injury, proliferate, and begin an immune response (Färber and Kettenmann, 2005). The microglia have various neurotransmitter receptors, including subtypes of glutamate, ATP, GABA, and norepinephrine receptors which, when activated, can cause internal Ca^{2+} to increase (Noda et al., 2000; Kuhn et al., 2004; Boucsein et al., 2003; Prinz et al., 2001). Interestingly, it has been shown that ATP released from astrocytes participating in a calcium wave causes responses in microglia (Verderio et al., 2001).

Glutamate and ATP in Neuron-Glia Signaling in Rat Optic Nerve

Application of ATP or glutamate in the neonatal rat optic nerve causes an increase in glial Ca^{2+} (Kriegler and Chiu, 1993; Butt et al., 2004). Electrical stimulation elicits a similar response as applying the ligands to the nerve (Kriegler and Chiu, 1993; James and Butt, 1999; Butt et al., 2004). However, the physiological role of each ligand for non-synaptic axon-glia signaling is still not entirely clear. In a study focusing on immunohistochemically identified synantocytes, Butt et al. (2005) examined the Ca^{2+} response to bath-applied ATP or glutamate and to electrical stimulation in optic nerve of P15 rats. They showed that NBQX, an AMPA receptor antagonist, blocked the Ca^{2+} increase in glial cells caused by applied glutamate, but not that caused by ATP. James and Butt (2000, 2001) showed that glial Ca^{2+}

also was increased in optic nerve glia by bath-applied ATP, ADP, and adenosine via activation of both ionotropic and metabotropic purinergic receptors. When suramin, an ATP receptor blocker, was applied, both the glutamate and ATP responses were blocked. Together, these findings suggest that ATP acts to increase glial Ca^{2+}, and that glutamate acts to stimulate ATP release. Butt et al. (2005) found that with electrical stimulation, suramin, but not NBQX, blocked the increase in glial Ca^{2+}, suggesting that under these more physiological conditions ATP is released from the axons and activates glia while glutamate may not be involved. It should be noted, however, that no other glutamate receptor blockers were tested, that they studied optic nerves from P15 rats, i.e. a developmental stage at which oligodendrocytes have differentiated and formed myelin, and, as explained later in this thesis, conditions may not have been optimal to detect involvement of glutamate. Recently, it has been reported that kynurenic acid, a broad spectrum ionotropic glutamate receptor antagonist, completely blocks the Ca^{2+} increase seen in oligodendrocyte cell bodies during ischemia (Micu et al., 2006). This suggests that glutamate is released during ischemia and causes oligodendroglial cell activation through ionotropic glutamate receptors.

ATP released from glial cells may signal more than just neurons. Extracellular ATP and intercellular IP_3 appear to play a role in glia-glia signaling. Guthrie et al. (1999) showed that ATP was present in the medium after calcium wave propagation, and purinergic receptor blockers and ATPases blocked the waves in cultured mouse cortical astrocytes. Conversely, cultured glioma cells not expressing gap junction proteins do not exhibit calcium waves, but the response is rescued with gap junction formation (Charles et al., 1992). Currently, it is not clear what the physiological role of the calcium waves are. However, it is suggested that they are involved in regulating neurotransmitter uptake and metabolism, growth and

proliferation, changes in cerebral vasculature, secretion of various neuroactive compounds, and modulating neuronal activity (Bell et al., 1990; Kim et al., 1994; Metea and Newman, 2006; James and Butt, 2001; Parpura et al., 1994; Fiacco and McCarthy, 2004).

NAAG as an Optic Nerve Signaling Agent

NAAG, in addition to glutamate, or instead of it, may be a non-synaptic intercellular signaling agent in rodent optic nerve. Tsai et al. (1990) showed that NAAG is released from rodent optic nerve terminals upon electrical stimulation of the nerve. If NAAG is a physiological extra-synaptic signaling agent, it could have an effect by acting on glutamate receptors directly or by being hydrolyzed to form glutamate. This is possible because GCP is present in the nerve as well (Sherman, Dobrin, Lubischer, and Grossfeld, unpublished data). Because NAAG acts on some of the same receptors as glutamate, it is difficult to distinguish whether either or both is a physiological signal or pathophysiological agent. To determine if NAAG is a physiological signaling agent in the optic nerve, it must be shown that 1) axons can release it, 2) glial cells respond to it and 3) an alteration in normal function is seen in the glial cells.

No work has been reported by other investigators to test whether NAAG is released non-synaptically upon electrical stimulation of the optic nerve. Extra-synaptic NAAG release has been shown only in crayfish CNS tissue. Similar studies must be conducted with optic nerve to determine if NAAG is an extra-synaptic axon-glia signal.

Changes in intracellular calcium levels in response to glutamate or NAAG can be monitored by calcium imaging. Through the use of calcium-sensitive fluorescent dyes, the spatial and temporal changes in calcium levels in individual cells can be detected and

measured. Focusing on a single cell over time, the change in fluorescence intensity indicates a change in intracellular calcium. Intracellular calcium is of great importance to all cells, including glia of the optic nerve. It plays a role, for example, in second messenger cascades, neurotransmitter release, and cell apoptosis. In general, calcium imaging is useful in determining both if a compound has an effect on a cell and what the functional outcome may be, especially for small cells whose activity is difficult to measure by other means. For glial cells, which do not generate electrical impulses, increases in cellular calcium represent a primary mode of cell excitability and intercellular communication. Due to the difference in conduction speeds of action potential propagation and calcium waves, calcium waves are likely to serve as a modulatory signal in the nervous system.

One example of a calcium-sensitive dye is Fluo-3 AM. It is essentially non-fluorescent in the absence of calcium and increases in fluorescence 40-fold when bound to calcium. An advantage of the Fluo-3 series of dyes is that they bind calcium comparatively more weakly than other calcium indicators. This allows for excellent imaging of transient calcium changes (http://store.mipcompany.com/fluo3.html). Another useful feature of these dyes is that they exist in the form of a lipophilic acetoxymethyl ester (AM-ester). This permits the dye to readily move into a cell, where it can be cleaved by cellular esterases to prevent its leak back out. As with all fluorescent dyes, photobleaching is a potential technical hurdle. When the dye is exposed to an excitation wavelength of light for an extensive period of time, the intensity of light emitted by the activated dye is reduced. This can be minimized by decreasing the intensity of the excitation light used (by passing it through a neutral density filter) and the exposure time.

It was the objective of this study to determine whether glutamate and NAAG fulfill

one of the 3 criteria for axon-glia signaling agents in neonatal rat optic nerve. Using Ca^{2+} imaging, I tested if glutamate and/or NAAG can activate glial cells in P5-9 rat optic nerve and initiated preliminary studies designed to ultimately test whether the effect of electrical stimulation involves glutamate and/or NAAG signaling.

Materials and Methods

Materials and Solutions

The green light emitting Ca^{2+} - sensitive fluorescent probe Fluo-3 AM was purchased from Molecular Probes (Eugene, OR). Stock solutions were prepared by adding 50µl anhydrous DMSO (Sigma-Aldrich; St. Louis, MO) and 0.5 µl 20% Pluronic F-127 in DMSO (Molecular Probes) to 50 µg of Fluo-3 AM. The following compounds were purchased from Tocris Bioscience (Ellisville, MO): N – acetylaspartylglutamate (NAAG), the AMPA/ group I mGluR agonist quisqualate, the group I/II mGluR agonist 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), the group II mGluR agonist 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC), AMPA, NMDA, the AMPA desensitization blocker cyclothiazide (CTZ), the glutamate uptake inhibitor DL-threo-ß-benzyloxyaspartate (TBOA), the AMPA/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) disodium salt, the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H- dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), the group I mGluR receptor antagonist 1-aminoindansign-1, 5-dicarboxylic acid (AIDA), the group II mGluR receptor antagonist (2S)-α-ethylglutamic acid (EGLU), and the group III/II mGluR receptor antagonist (RS)-alpha-cyclopropyl-4-phosphonophenylglycine (CPPG). ATP was from Fisher Scientific (Fair Lawn, NJ), and glutamate, aspartylglutamate, *N*-acetyl-β-aspartylglutamate (ß-NAAG), N-acetylaspartate, and crude collagenase Type IA

(from *Clostridium histolyticum*) from Sigma-Aldrich. The GCP inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) was a gift from Guilford Pharmaceuticals Inc. (Baltimore, MD), now known as MGI Pharma.

Artificial CSF (αCSF), modified from previous studies (James and Butt, 1999) to remove GCP-II inhibiting phosphate, contained (in mM) NaCl 134.2, KCl 3, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, and HEPES 10. It was brought to pH 7.3 with 10 mM NaOH and oxygenated with 100% O_2 in all cases. Nominally Ca^{2+}-free αCSF was made by replacing $CaCl_2$ by equimolar $MgCl_2$. In experiments in which 1.2 mM NaH_2PO_4 was included in the αCSF with the intent of inhibiting GCP, NaCl was reduced to 133 mM. All chemicals were prepared in oxygenated αCSF and experiments were performed at room temperature (23 - 25°C)

Nerve Preparation

Wistar rats, obtained from Charles River Breeding Laboratories (Raleigh, NC), were housed and bred in the Biological Research Facility at North Carolina State University. At postnatal day 5-9 (P5-9), rats were euthanized by overdose of isoflurane, in accordance with an approved IACUC protocol (04-154B; P.I. Jane Lubischer) and NIH guidelines for the care of laboratory animals. They were decapitated to isolate the optic nerves from behind the orbit to the chiasm, approximately 3-5 mm in length. To facilitate removal of the dura and pia layers, the nerves were incubated for 0.5 h in 1 ml αCSF containing 0.1% collagenase (343 units collagen digesting activity per ml). They were then separated at the chiasm and their meninges mechanically removed. Both nerves were incubated together in 1 ml αCSF containing 7.5 µl Fluo-3 AM. After 1.5 h, the nerves were transferred to fresh αCSF for 0.5 h to allow excess dye that had not been internalized by cells to be washed away. The nerves

were kept in this solution until use – 0.5 h for the first nerve and 1.5 h for the second. Roughly three quarters of the time nerves from the same animal did not give the same response (i.e. number of cells with un-stimulated and stimulated Ca^{2+} increases). Sometimes the first nerve had more cells responding than the second but other times the reverse was true. The differences in response may be attributed to slight differences in desheathing and handling of the nerves.

Imaging Chambers

Different imaging chambers were designed for experiments involving neurotransmitter receptor activation by exogenous drugs or electrical stimulation. Each chamber was constructed to minimize the bath volume as well as ensure rapid fluid exchange when switching between solutions. Three 50 ml syringes held test solutions and one held saline, with the outflow 10 cm above the microscope stage. They were punctured near their base with 18 gauge syringe needles to allow oxygenation. Polyethylene tubing (1.15 mm i.d.) led to a 6 input–1 output mechanical switch, which controlled flow into either imaging chamber.

A modified Warner Instruments (Hamden, CT) model PH6 series 20 chamber was used for application of neurotransmitter receptor agonists. It had a single bath compartment with a layer of silicone glue applied to the base to reduce the total bath volume to approximately 0.5 ml. Using a gravity flow system, the flow rate of αCSF was held at 2 ml/min. Test solutions were made up in differing total volumes (4-10 ml), and thus had lower flow rates (1– 2 ml/min). Consequently, depending on the volume in the syringe, it took approximately 25 – 45 sec for complete turnover of the chamber contents when switching between solutions, measured visually by the amount of time taken for water containing red

food coloring to be replaced by water containing blue food coloring. The nerve was placed in the center of this chamber and was held stationary during experimentation by a U-shaped platinum wire with nylon strands glued across it.

The chamber for electrical stimulation (Fig. 3) was made by gluing pieces of glass microscope slides onto a glass base. Two channels were created. One channel (the vertical one in Fig. 3) was used to flow solutions past the nerve. The total volume of this channel was approximately 25 µl. This created an approximately 10 sec delay for complete turnover when switching between solutions. The nerve was placed in the second channel (the horizontal one in Fig. 3) so that its center crossed the vertical flow channel. A small amount of Dow Corning 3 silicone grease was used to hold the nerve in place at the edges of the flow channel. Additionally, these grease barriers effectively divided the chamber into three electrically isolated sections. Silver/silver chloride wires were placed in the left and central sections. They were used to electrically stimulate the nerve. Two more Ag/AgCl wires were similarly placed between the central and right sections of the chamber to record compound action potentials (CAPs). The recording wire placed in the central section of the chamber was connected to ground. A glass cover slip was placed on top of the entire chamber and secured with silicone grease to ensure that no contact occurred between the solutions in the separate sections. Because of space and electrical constraints, the nerve was not weighted down in the electrical stimulation chamber. This caused the nerve to drift slightly during imaging trials. This was corrected for when analyzing images of cellular responses.

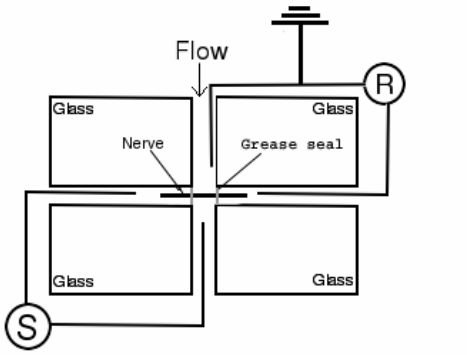

Figure 3. Schematic of chamber used for electrical stimulation. The chamber was 40 mm long and 20 mm wide. The spaces between the glass pieces were 1.5 mm. Grease seals were used to form 3 electrically isolated compartments. The nerve (3 -5 mm long) was stimulated by passing suprathreshold current pulses from a wire in the left-most compartment to one in the center compartment. CAPs were recorded between a wire in the right-most compartment and a grounded wire in the center compartment.

Electrical Stimulation

For experiments involving electrical stimulation, suprathreshold AC pulses were delivered from a Grass S44 stimulator via a Grass SIU5A stimulus isolation unit. Stimulation pulses were 10 – 15 V and 0.1 – 0.3 ms duration, at 20Hz. Compound action potentials were recorded on a Tektronix 5110 oscilloscope through a Grass AC/DC strain gage P122 amplifier.

Calcium Imaging of Glia

For both experimental designs, the chamber was placed on the stage of an upright Leica DM R microscope with epifluorescence optics. The nerve was immersed in solution at all times during placement in the chamber and secured using either platinum wire or silicone grease. A 20x HCX APO 0.50 NA water immersion objective and a mercury bulb filtered to allow an excitation wavelength range of 450 – 490 nm and emission wavelength range of 500 – 550 nm were used to image the changes in fluorescence of the dye in glial cells, which appeared as punctate spots (James and Butt, 1999). αCSF was continuously superfused through the chamber at all times unless otherwise noted. Because of the circular nature of the nerve and non-confocal microscopy, it was difficult to focus on the entire surface of the

nerve at once. Also, it was only possible to image glial cells near the surface with this technique.

In all experiments, the nerve was first superfused with αCSF for 2 min to obtain a baseline recording. Light emission from some cells occurred spontaneously ("oscillated") without external stimulation or drug treatment. This interval allowed identification of oscillating cells and approximate frequency of oscillation. To test the effect of exogenous neurotransmitter receptor agonists, the nerve was superfused first with αCSF for 20 – 90 sec, and then with αCSF containing NAAG, glutamate, or ATP. Drug application was continued for 1 min, after which superfusion with αCSF was resumed and fluorescence recorded for an additional 1 – 3 min. For several experiments, NAAG was applied for 30 sec and then the flow was stopped for 1 min. The intent was to allow time for formation and accumulation of glutamate during the enzymatic hydrolysis of NAAG. In experiments involving treatments of this sort, control treatments included an equivalent 1 min stoppage of flow.

To test the effect of electrical stimulation, the nerve was first superfused with αCSF and then the flow was stopped for a 60 sec period of high frequency (20 Hz) stimulation. Flow was stopped to allow any released chemicals to accumulate extracellularly. Then, stimulation ceased and the flow of αCSF was resumed. Imaging of the nerve continued for 1 – 5 min to determine whether there were any responses that persisted after stimulation ceased. For some trials, treatment with CTZ plus TBOA or with 2-PMPA was performed for 2 – 10 min before and during electrical stimulation. CTZ, an AMPA receptor desensitization blocker, and TBOA, a glutamate reuptake inhibitor, were used together to increase the effectiveness of extracellular glutamate in activating glutamate receptors. 2-PMPA, a specific inhibitor of the GCP enzymes, was used to test whether intact NAAG activates optic

nerve glia.

For each nerve, several successive trials were performed. The following general protocol was used. First, a control trial with αCSF was performed (with stopping the flow when appropriate). Next, the experimental treatment was performed. A 10 min "recovery" period followed, during which αCSF flowed over the nerve, but no images were taken. The intent was to allow sufficient time for cellular calcium levels to return to resting levels while minimizing photobleaching. The experimental treatment was then repeated, followed by another 10 min recovery period. Finally, the nerve was treated with either glutamate or ATP to determine whether it was still responsive and to provide a reference for comparison of various treatments.

Purification of NAAG

To ensure that any acidic contaminants, especially glutamate and aspartate, were removed, NAAG was purified using cation exchange mini-columns (0.6 cm x 2 cm, AG50WX4, 200 – 400 mesh, H^+ form; Bio-Rad, Richmond, CA). The columns were first rinsed with water and then equilibrated with 0.1 M HCl. Then, 100 µl of 100 mM NAAG, dissolved in water, were applied to the column resin. The unbound liquid was collected with two 1 ml 0.1 HCl rinses. This NAAG fraction was dried under vacuum at low heat in a Savant Spin Vac SC110. The residue was resuspended in water twice and dried as previously. The residue was finally resuspended in 4 ml of αCSF for application to the nerve at a final concentration of 2.5 mM. A control column was treated similarly with a sample of water.

To check recovery of NAAG and removal of glutamate, two additional columns were treated with 0.2 µl H^3-NAAG or 0.2 µl H^3-glutamate. NAAG was eluted as above and

glutamate was recovered with two 1 ml volumes of HCl. Radioactivity was measured in 5 ml Ecolume liquid scintillation cocktail (MP Biomedical; Irvine, CA) in a Beckman LS5100 counter.

Data Analysis

Images were digitally recorded at a rate of 1 image per second with Openlab 3.5.1 software (Improvision; Lexington, MA) on an Apple Macintosh G4 PowerMac computer running Mac OS X 10.2.4. Individual glial cells were selected by visual inspection based on a punctate spot of increased fluorescence. The Openlab software was further used to measure the maximum intensity of each cell selected and quantitate the fluorescence over time. Care was taken to ensure the responses were from the same cells. The intensity measurements were then collected in Microsoft Excel and a rolling average was determined for each measurement to reduce the effect of random noise. JMP 5.1 was used to graph each cell's intensity change over time. Because the rolling average would obscure any transient spikes, both the raw data and rolling average were examined. In each nerve, approximately 50 – 200 cells were analyzed. Between 4 and 10 nerves, from 2 – 5 different animals, were examined per experiment.

To assess the effect of receptor agonists or antagonists on the activity of glial cells, the data from the pre-treatment control period and the following experimental treatment period were divided into 5 sec bins and the total number of cells exhibiting increased fluorescence during those times tallied. A cell was considered to be responding for a given time bin if its fluorescence was greater than 100 units above the cell's individual baseline during that bin. Although this approach to analyzing the data has the disadvantage that spontaneous oscillations will be included along with drug-related responses, it does allow

identification of the latter in terms of either an increase or decrease in the number of active cells during drug treatment in comparison with the preceding control period. Another disadvantage of this approach is that the qualitative aspects of an individual cell's Ca^{2+} increase, such as whether it is a spiking or sustained response, is ignored. This approach also may under-represent the total number of cells responding during the course of an experiment. If, for example, a drug were to cause 100 cells to increase in calcium simultaneously, the response would be seen more dramatically than if it had caused the same 100 cells to increase over a more prolonged time period. Where indicated, the data are presented as the mean number of cells responding ± S.E.M.

Results

Calcium Transients Occur Spontaneously in Optic Nerve Glia

As previously reported (Fatatis and Russel, 1992; Kriegler and Chiu, 1993; Kim et al., 1994; Parri et al., 2001), spontaneous increases in calcium were seen in unstimulated nerves (Fig. 4). This occurred in as many as 50% of the cells that exhibited increased fluorescence during chemical treatment or electrical stimulation. Two general types of spontaneous fluorescence were seen - random and repetitive. About three-quarters of the oscillating cells showed random increases (Fig. 4A), which typically were single Ca^{2+} spikes with long (i.e. greater than 2 min) intervals between them. About one-quarter of the spontaneously active cells showed repetitive oscillations (Fig 4B), characterized by a sequential increase in Ca^{2+} with short (i.e. <5 sec) gaps in time between spikes. The number of cells with non-stimulated increases in Ca^{2+} rose from approximately 5 – 15 per nerve to approximately 15 – 80 per nerve by avoiding even brief exposure to air by carefully using a plastic transfer

pipette and keeping the nerve submerged in αCSF at all times. This implies that the oscillations are a sign of viable glia, rather than of damaged cells.

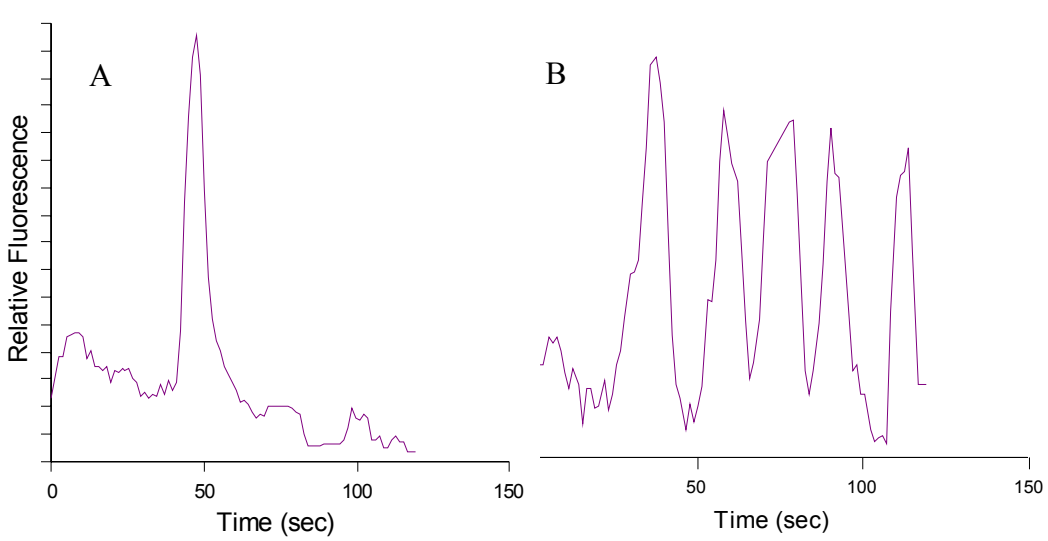

Figure 4. Spontaneous Ca^{2+} changes in glia of unstimulated optic nerve. During a control treatment (no receptor agonists applied), some optic nerve glia show a spontaneous Ca^{2+} increase. *A* shows a cell which increases a single time – termed random. *B* shows a repetitive oscillator which increases intracellular Ca^{2+} many successive times with short gaps of time between Ca^{2+} increases. *A* and *B* were taken from different cells in the same nerve during the same control trial.

The spontaneous Ca^{2+} increases were not eliminated in a nominally Ca^{2+}-free saline or when the nerve was incubated for 3 min with a cocktail of purinergic receptor blockers (i.e. 10 μM CGS 15943 for P1 adenosine receptors and 100 μM suramin for P2 ATP receptors). As a result, it was difficult to distinguish an experimental response from a naturally occurring oscillation. Thus, a nerve response to an experimental treatment was defined as an increase in the number of cells showing an increase in Ca^{2+}. It is possible that responses may be occurring at lower concentrations than measured but were not able to be distinguished from the natural oscillations. A typical response to bath-applied chemicals had 20 to 150 more cells with increased Ca^{2+} than during the preceding control period.

Glutamate Activates a Glial Ca^{2+} Response

Consistent with previous reports (Kriegler and Chiu, 1993; Butt and Tutton, 1992;

Butt et al., 2005), I found that exogenous glutamate application to the rat optic nerve causes a calcium increase in the glia. Glutamate was effective in increasing glial cell calcium in a concentration-dependent manner from 50 μM (Fig 5). When glutamate was applied in the presence of TBOA to block its cellular uptake and of CTZ to block AMPA receptor desensitization, the sensitivity to glutamate was enhanced and the response occurred sooner and was prolonged (Fig 6). A lower concentration, 10 μM, of glutamate increased intracellular calcium after a 2 min preincubation with 100 μM CTZ and 50 μM TBOA than without these chemicals present. At least a portion of the increase in Ca^{2+} was from intracellular sources. Glutamate (100 μM) activated a glial calcium response in nominally Ca^{2+}-free αCSF, typically starting 30 - 60 sec after application. In αCSF without TBOA or CTZ, 40 μM NBQX (an AMPA receptor blocker) reduced the number of cells responding to 100 μM glutamate by approximately one-quarter and delayed the response by approximately 30 sec as compared to that with glutamate application alone.

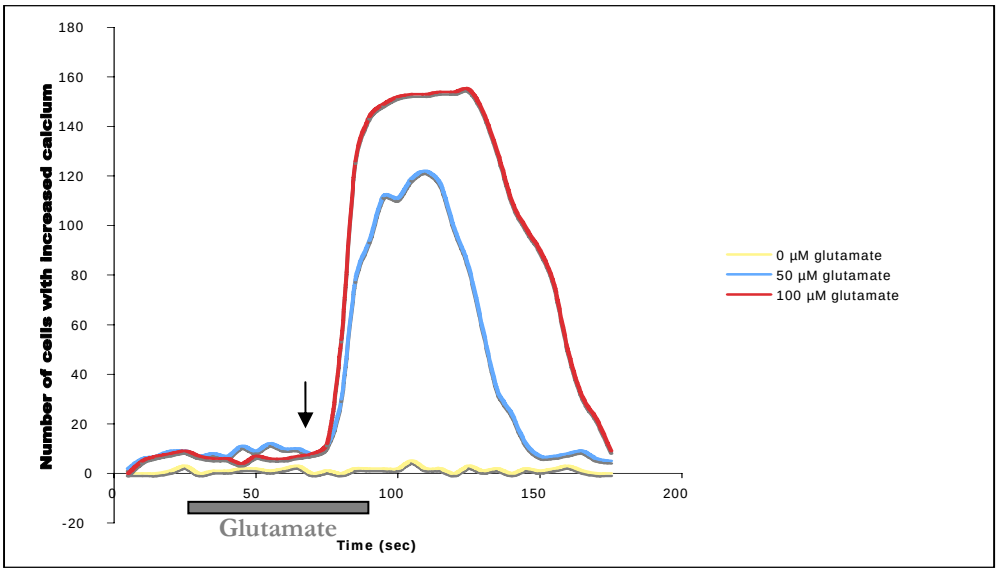

Figure 5. Dose-dependent response of glia to glutamate. For each trial, glutamate was bath-applied from 30 – 90 sec. The arrow represents the calculated time at which glutamate would be expected to reach the desired concentration in the imaging chamber. The bar represents (in all figures) the time during which glutamate was allowed to flow into the chamber. Time zero indicates when image acquisition for each trial began. There were a greater number of cells responding to 100 µM glutamate than to 50 µM glutamate. Even though both responses began at approximately the same time, the response to 100 µM glutamate was longer than that to 50 µM. This graph represents a whole nerve response in which the number of cells with increased Ca^{2+} is plotted in two successive trials and is representative of the 4 nerves (from 2 animals) tested. The control treatment (0 µM glutamate) shows the baseline number of cells in this nerve fluorescing spontaneously without glutamate application.

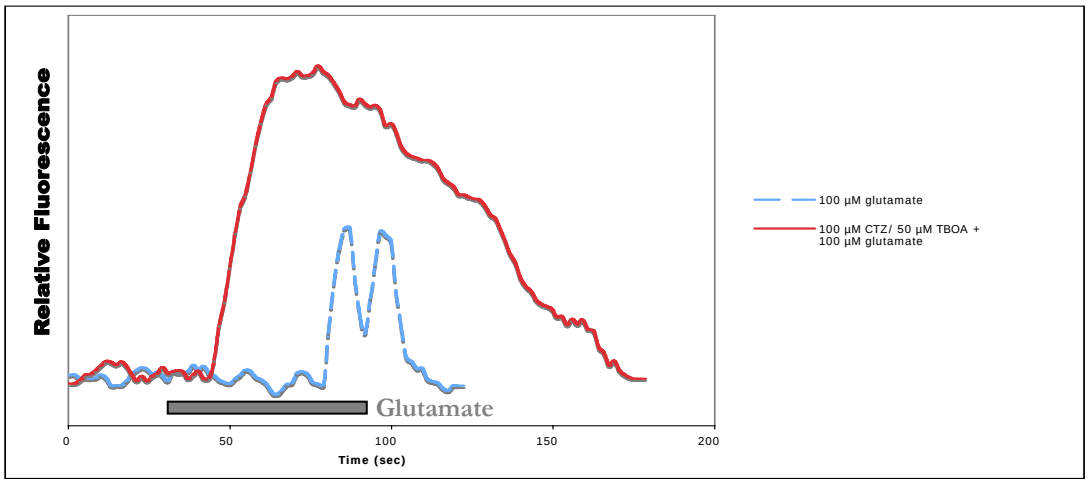

Figure 6. The glial Ca^{2+} response was prolonged and earlier with CTZ and TBOA. For both treatments, 100 µM glutamate was applied for 60 sec. The treatment represented by the solid line included a CTZ and TBOA preincubation for 2 min (not shown) and then continued treatment with those compounds and 100 µM glutamate for 60 sec.

Because of its purported role in axon-glia signaling, ATP was commonly used in these experiments as a reference. Nearly three-quarters (70±2%) of the total observed cells (9 nerves, 577 total cells, minimum of 48 cells per nerve) which responded to 100 μM glutamate also did to subsequent 100 μM ATP application. However, 5% (5±3%) of the total observed cells (9 nerves, 577 total cells, minimum of 48 cells per nerve) responded to ATP but not to glutamate. The remaining ~25% of cells that responded to glutamate but not subsequently to ATP may represent cells that are glutamate- but not ATP-sensitive or may reflect a change in sensitivity of the cells with repeated trials (discussed further below in Responses to Successive Glutamate Treatments are Similar but not Identical). Glial calcium activation produced by ATP occurred at a lower concentration, 10 μM, than that produced by glutamate alone.

Glutamate Receptor Agonists Activate a Glial Ca^{2+} Response

Selective glutamate receptor agonists were applied to the nerve to determine which receptors may be activated by glutamate. Quisqualate (an AMPA/mGlu I receptor agonist), AMPA, NMDA, ACPD (an mGluR I/II agonist), and APDC (an mGluR II agonist) were separately applied at 100 μM for 1 min. Each of these compounds caused an increase in glial Ca^{2+}, except for NMDA. It has been previously reported that NMDA causes a Ca^{2+} increase in OPCs in culture (Wang et al., 1996). The likely reason for differences in results between this study and Wang et al. is that Wang et al. co-applied NMDA with glycine in a Mg^{2+}-free medium while I applied NMDA without exogenous glycine in a Mg^{2+}-containing saline. Kriegler and Chiu (1993) showed a glial response in both a normal and Ca^{2+}-free bath, and showed the response existed in a spiking and sustained form. Also, they separately applied AMPA and ACPD and showed a glial Ca^{2+} increase with both. They concluded that both

ionotropic and metabotropic receptors were involved in the glutamate response. The specific glutamate agonist data presented here supports and expands upon that conclusion in showing which specific categories of glutamate receptors may be involved, namely AMPA and group I and II metabotropic receptors.

Responses to Successive Glutamate Treatments are Similar but not Identical

To test for reproducibility of responses and so experimental treatments could be accurately compared, successive trials were conducted on each nerve. Preliminary work showed that approximately 80% (80±2%) of the cells (10 nerves, 598 total cells, minimum of 21 cells per nerve) which responded to one glutamate application also did to the next. These data suggest that although some decrease in response would be expected with successive treatments, the majority of cells responding in one trial would be expected to respond similarly in the following trial on the same nerve. It also was found that successive treatments with 1 mM glutamate caused 60% (60±1%) of the cells (10 nerves, 598 total cells, minimum of 21 cells per nerve) responding in one trial to increase the time to peak fluorescence in the subsequent trial.

NAAG Causes an Increase in Glial Calcium that may be Partially Due to its Hydrolysis to Form Glutamate

Application of NAAG was effective in increasing glial calcium at concentrations as low as 2.5 mM. The typical glial response to 2.5 mM NAAG was a spiking increase in intracellular Ca^{2+} (Fig. 7A), whereas 5 mM NAAG caused glial calcium to stay elevated for a longer period (Fig. 7B). When 2.5 mM NAAG was applied, an inflection in the graph of number of cells responding vs. time suggested that there were multiple distinct populations of responding cells in 9 out of 15 nerves (Fig. 8). Preincubating the nerve with CTZ and TBOA

made it possible to see a response at a lower concentration, 1 mM NAAG (9 out of 9 nerves) and 100 µM NAAG (8 out of 10 nerves). These results suggest that some NAAG was hydrolyzed to form glutamate, and that the sensitivity for detecting its activation of AMPA receptors was enhanced by addition of CTZ and TBOA to the CSF.

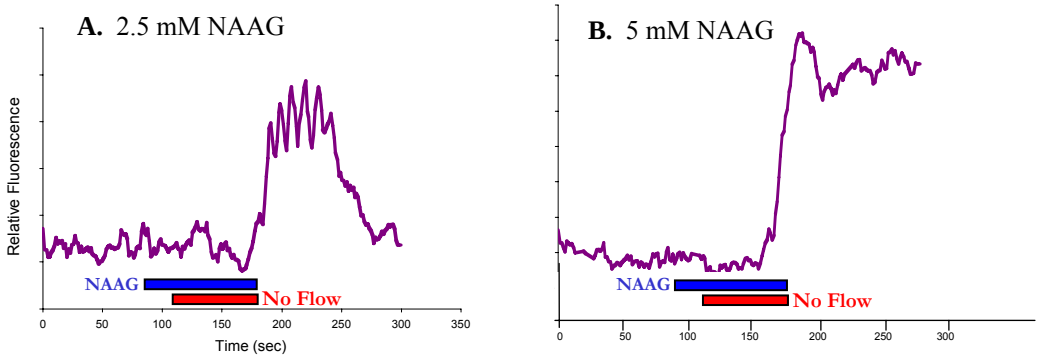

Figure 7. Dose-dependent response of glia to NAAG. *A* shows a typical glial response to 2.5 mM NAAG. NAAG was applied from 90 – 120 sec and then the flow was stopped from 120 – 180 sec. *B* shows a typical response to 5 mM NAAG. NAAG was applied in the same manner as for the cell in *A*. The 5 mM response occurred slightly earlier and lasted longer.

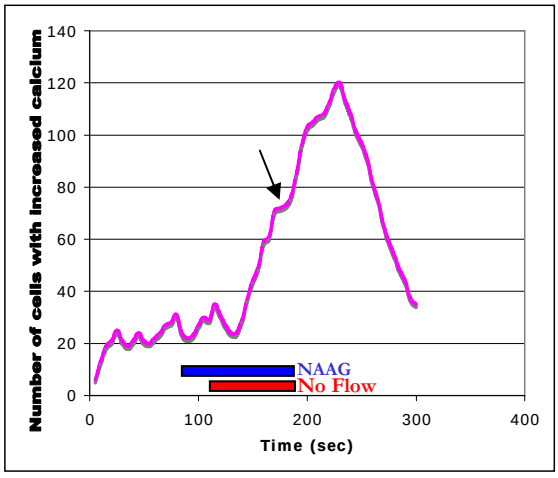

Figure 8. NAAG may activate multiple populations of glia. 2.5 mM NAAG was applied to the nerve from 90 – 120 sec and then the flow was stopped from 120 – 180 sec. This graph represents the number of cells responding within each 5 sec. The first cells responding typically increased in fluorescence starting at approximately 50 - 80 sec after the initial NAAG application. Approximately 30 – 60 sec later (arrow), an inflection in the graph suggests that a second population of glia began to show an increase in calcium as well.

No increase in the number of cells with increased calcium was seen above the spontaneous oscillations when the nerve was exposed to αCSF alone or to 2.5 mM aspartylglutamate. However, N-acetylaspartate, which would be formed during the

hydrolysis of NAAG, when applied at 2.5 mM, did cause an increase in glial Ca^{2+}. To be certain the effect seen by NAAG application was not via a glutamate contaminant, NAAG was purified using ion exchange chromatography. The purified NAAG was then applied to the nerve and a response was seen at 2.5 mM.

Intact NAAG Activates a Glial Ca^{2+} Response

To test whether NAAG causes a glial Ca^{2+} increase independent of glutamate formation, two approaches were taken. First, a non-hydrolyzable form of NAAG, ß-NAAG, was applied to the nerve. A response was seen at 2.5 mM. However, in 3 of 4 nerves tested (from 3 animals), at the peak of the response 50 – 70 % fewer cells responded than to NAAG and the response began 25 – 55 sec earlier (Fig. 9). This was true no matter if the NAAG trial preceded or followed ß-NAAG application. However, in those nerves, the area under the curve of the ß-NAAG response was greater than or equal to the area under the curve of the NAAG response. An increase may represent a difference in the number of responding cells or in the duration of their response. As a second test of whether intact NAAG can activate optic nerve glia, the nerve was preincubated with 10 μM 2-PMPA, a GCP inhibitor, in αCSF containing phosphate, also known to inhibit GCP activity, and then was treated with NAAG and 2-PMPA in phosphate-containing αCSF (Fig. 10). A response was still seen with 2.5 mM NAAG when inhibiting GCP in these two ways. This shows that NAAG itself can cause an increase in glial Ca^{2+} without its hydrolysis to form glutamate and NAA.

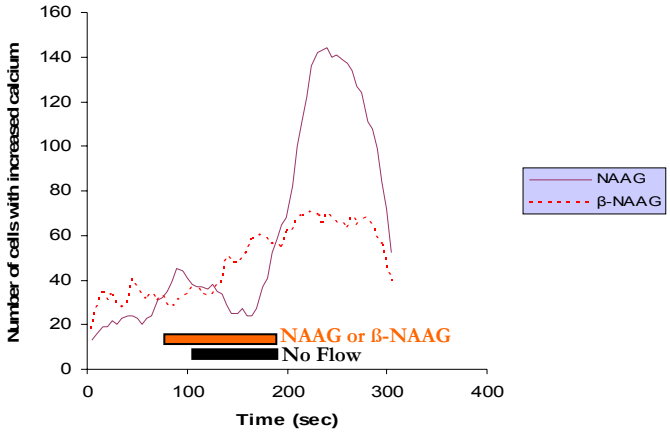

Figure 9. Glia respond to ß-NAAG. 2.5 mM ß-NAAG or NAAG was applied from 90 – 120 sec and then the flow was stopped from 120 – 180 sec. The initial response to ß-NAAG occurred approximately 25 sec before the response to NAAG. In 3 of 4 nerves tested, the peak of the response had 50 – 70% fewer cells responding to 2.5 mM ß-NAAG than to 2.5 mM NAAG regardless of the order of the trials.

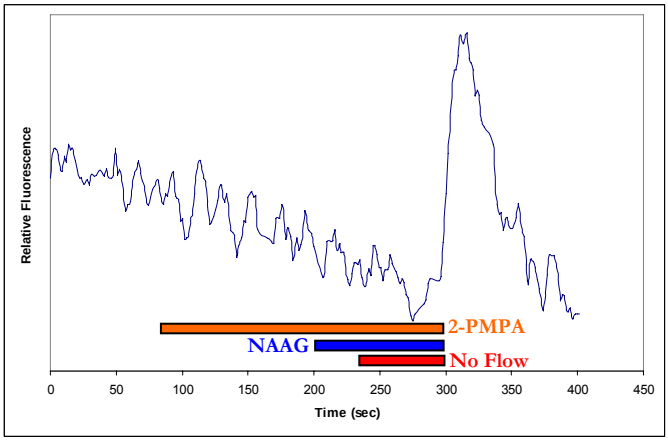

Figure 10. NAAG with the GCP inhibitor 2-PMPA elicits a Ca^{2+} increase in optic nerve glia. After preincubation with 10 µM 2-PMPA for 120 sec, 2.5 mM NAAG together with 10 µM 2-PMPA was applied to the nerve 30 sec before stopping the flow for 60 sec to allow accumulation of any NAAG hydrolysis products. An increase in glial calcium was seen in 4 out of 4 nerves tested from 2 animals.

Electrical Stimulation Activates a Glial Ca^{2+} Response

Electrical stimulation of the optic nerve at 20 Hz induced an increase in glial Ca^{2+}. When the nerve was preincubated with CTZ and TBOA and then electrically stimulated, in 5 (from 5 different animals) of 35 (from a total of 20 different animals) rat optic nerve preparations the number of responding glial cells increased. Movement of the nerve in the electrical stimulation chamber resulting from fluid flow through a small, sealed compartment may have caused the nerve to be stimulated unequally in successive trials based

on changes observed in the nerve's compound action potentials. Because of this, it was difficult to determine if the increase in number of active cells with stimulation in the 5 nerves after incubation with CTZ and TBOA was real or not. Further studies must be conducted with alterations in the procedure to keep the nerve stationary.

Discussion

Ca^{2+} Oscillations Occur in Some Glia Without Stimulation of Optic Nerve

Without electrical or chemical stimulation a large number of cells showed an increase in intracellular Ca^{2+}. Charles et al. (1991) showed that mechanically stimulated glial cells in culture produce an asynchronous Ca^{2+} response. It is possible that the act of isolating the nerve and desheathing it may mechanically stimulate the nerve and cause the Ca^{2+} increases observed. However, natural Ca^{2+} transients have been reported by many investigators using a variety of approaches (Fatatis and Russel, 1992; Kriegler and Chiu, 1993; Kim et al., 1994; Parri et al., 2001) and, therefore, may be influenced, but not likely caused by, the nerve isolation and desheathing procedures used here. In rat thalamus (Parri et al., 2001) and hippocampus (Nett et al., 2002), astrocytes generate increases in intracellular Ca^{2+} independently of neuronal activity. The Ca^{2+} increase is linked to release from internal stores and probably unrelated to glutamate or purinergic receptors or vesicularly released chemicals (Parri et al., 2001; Nett et al., 2002; Parri and Crunelli, 2003). Hirase (2004) made similar observations *in vivo* in the rat cortex. The increases in glial Ca^{2+} persist in the presence of agents which block neuronal activity (Zur Nieden and Deitmer, 2006; Fiacco and McCarthy, 2006) Therefore, they may be referred to as "intrinsic" Ca changes rather than as changes provoked by an external stimulus (Fiacco and McCarthy, 2006).

Parri et al. (2001) reported a neuronal inward current correlated with the spontaneous astrocyte activity, suggesting that astrocytes continually communicate with neurons. Increasing astrocytic Ca^{2+} causes glutamate release, which activates NMDA receptors on hippocampal neurons in culture (Araque et al., 1998a). The spontaneous astrocytic Ca^{2+} oscillations may cause glutamate release that then signals nearby cells. It has also been reported that the oscillations in astrocytes may be linked to changes in arteriole blood flow (Zonta et al., 2002). Therefore, these apparently spontaneous Ca^{2+} oscillations may be a physiologically relevant and important feature of the system.

NAAG Activates a Calcium Response in Optic Nerve Glia in its Intact Form and via its Hydrolysis Product(s)

Application of NAAG to neonatal rat optic nerve caused an increase of Ca^{2+} in glia. Both ß-NAAG and NAAG with 2-PMPA also caused an increase in glial Ca^{2+}. Thus, intact NAAG can activate optic nerve glia. In mammalian CNS tissue, intact NAAG is an agonist of type II metabotropic glutamate receptors (Wroblewska et al., 1993; Bischofberger and Schild, 1996), and a weak agonist of NMDA receptors (Valivullah et al., 1994). Application of NMDA did not elicit a response in optic nerve glia in my experiments but has been reported to do so by other investigators (Wang et al., 1996; Micu et al., 2006). It is unlikely that intact NAAG is causing a Ca^{2+} increase via NMDA receptors in rat optic nerve at the developmental stages and under the conditions that I studied. It is more probable that it is acting through mGluRs, possibly type II mGluRs. This is supported by the fact that application of APDC, a type II mGluR agonist, caused a glial Ca^{2+} increase. The response to 2.5 mM NAAG was seen in both normal αCSF and nominally Ca^{2+}-free αCSF, suggesting a metabotropic receptor component of the response. Either, contrary to the current literature

(Valivullah et al., 1994), NAAG can activate non-NMDA ionotropic receptors, or, more likely, glutamate formed by its hydrolysis is doing so in rat optic nerve glia. Applied NAAG may activate optic nerve glia through its hydrolysis by the ectoenzyme GCP, forming glutamate and NAA. It has been shown previously (Kriegler and Chiu, 1993; Butt et al., 2005), as well as in this study, that optic nerve glia respond to glutamate application. It is not only possible, but likely, that a portion of the response to NAAG seen in this study is through the formation of glutamate. When the nerve was preincubated with CTZ and TBOA to increase the effectiveness of any formed glutamate in activating receptors, a response to NAAG was seen at a lower concentration. This increase in sensitivity could be attributed to three possible mechanisms. First, the effectiveness of NAAG could be increased by CTZ blocking the AMPA receptor desensitization. The literature does not support an effect of NAAG on AMPA receptors. At AMPA receptors, NAAG is 700 times less potent than glutamate, with an IC_{50} of 7.9×10^{-4} (Valivullah et al., 1994). It is still possible, however, that NAAG may weakly activate AMPA receptors and, when in the presence of CTZ, the response becomes more evident. A second possibility is that TBOA, in addition to blocking glutamate uptake, also blocks NAAG uptake. A high affinity NAAG transporter was found in mouse neuron-glia co-culture (Cassidy and Neale, 1993a). Quisqualate inhibited the transport (in addition to inhibiting GCP activity) (Cassidy and Neale, 1993a). However, an effect of TBOA on the uptake of NAAG has not been reported. A third possibility is that the metabolite glutamate is formed from NAAG and, in the presence of CTZ and TBOA, elicits a response to NAAG at a 1 mM or lower concentration. It follows then that 1 mM NAAG may be eliciting a response with CTZ and TBOA present at least in part via glutamate formation and activation of AMPA receptors. However, optic nerve glia also respond to bath-applied

NAA, which has been shown to activate metabotropic glutamate receptors (Yan et al., 2003), so this cannot be ruled out as a possible factor in NAAG's action.

When 2.5 mM aspartylglutamate was applied to the nerve, no increase in glial Ca^{2+} was seen. Aspartylglutamate is hydrolyzed by purified GCP-II 4 times more readily than NAAG (Serval et al., 1990). If NAAG activates a glial Ca^{2+} increase through hydrolysis to form glutamate, then aspartylglutamate would be expected to act similarly. Since this was not found, it is possible that in intact nerve the breakdown of aspartylglutamate is reduced compared to its hydrolysis by the purified enzyme.

A Relatively High Concentration of NAAG was Necessary to Activate Increased Glial Ca^{2+}

To see a response from exogenous NAAG, a concentration greater than 1 mM was necessary. This concentration *in vivo* may never be reached or it possibly represents a pathological condition. A high NAAG concentration may be necessary to see a glial response because of high background Ca^{2+} oscillations or rapid uptake of glutamate, NAAG, or NAA.

It is possible that a lower concentration of NAAG may elicit a response in some optic nerve glial cells, but the high rate of background oscillations may mask it. A number of approaches were unsuccessful in eliminating or reducing the spontaneous oscillations, such as lowering external Ca^{2+} or incubating with purinergic receptor blockers. It is possible that the cellular uptake of intact NAAG or its metabolic products may cause the local concentration to be significantly lower. In mammalian brain, specific uptake mechanisms exist for NAAG, glutamate, and NAA (Cassidy and Neale, 1993, Logan and Snyder, 1971; Fagg and Lane, 1979; Sager et al., 1993). The uptake of these chemicals reduces the action of exogenous NAAG or its hydrolysis products on cellular receptors by lowering the extracellular concentrations. When the nerve was preincubated with CTZ and TBOA, a

lower concentration of NAAG was effective in eliciting a glial response. Since TBOA is known to block glutamate uptake, glutamate produced by NAAG breakdown may be responsible for at least a portion of the NAAG response. If only 1-10% of applied 1-2.5 mM NAAG were hydrolyzed under the conditions of my study, that would produce a concentration of glutamate (i.e. 10-250 μM) in the range that was detectable and that is consistent with estimates of extracellular glutamate concentrations during physiological stimulation (Kreigler and Chiu, 1993; Zur Neiden and Deitmer, 2006). Whether TBOA might also affect NAAG uptake has not been reported. Alternatively, or complementary, the increased effectiveness of NAAG seen after a CTZ and TBOA preincubation may be from CTZ maintaining AMPA receptors in an open state. If intact NAAG can weakly activate AMPA receptors, which is not expected based on previous studies, CTZ may be responsible for the response seen with a lower concentration of NAAG by prolonging AMPA receptor activation.

NAAG May Activate a Calcium Response in Multiple Populations of Glia

In 9 out of 15 nerves examined, the response to exogenously applied NAAG appeared to occur in two phases. First, there was a small increase in the number of responding cells, and then 30 - 60 sec later the number of cells responding increased further. This may result from an intrinsic difference in sensitivity between glial cell types activated by the same receptors or via diverse receptor types on different glial cells being activated at disparate times. Rat optic nerve glia are a heterogeneous mix with different properties. It is possible that intact NAAG activates mainly metabotropic receptors on some glial cells while glutamate formed from its hydrolysis then activates ionotropic receptors on other glia.

The Increase in Intracellular Ca^{2+} Probably Occurs in Both Astrocytes and Oligodendrocyte Precursor Cells (OPCs)

The optic nerve is an excellent model to study axon-glia interactions because it lacks neuronal somata. An increase in fluorescence in a punctate spot is indicative of a glial cell body. Kriegler and Chiu (1993) compared the morphology of optic nerve cells stained with calcium dye to that of optic nerve cells stained with a vital dye. They concluded that most of the cells stained with the calcium dye, which are round and randomly distributed, were not endothelial cells, which appear thin and tend to form tandem lines along capillaries. Of the glial cell types present in the adult rat optic nerve, astrocytes, oligodendrocytes, synantocytes, and microglia all are known to express functional glutamate receptors (Bell et al., 1991; Steinhäuser et al., 1996; Fulton et al., 1992; Noda et al., 2000). Furthermore, glutamate has been shown to cause an intracellular Ca^{2+} increase in all four cell types (Butt et al., 2004; Butt et al., 2005; Light et al., 2006).

Approximately 85 – 90% of the neonatal optic nerve glial cells are astrocytes or oligodendrocytes precursor cells (OPCs) (James and Butt, 2001). In both there is an increase in intracellular Ca^{2+} upon application of glutamate (Steinhäuser et al., 1996; Bell et al., 1991). It is most likely, then, that these cells are the majority of the cells being observed in this study. However, synantocytes comprise 7% of the glial cells in the mature rat optic nerve (Butt et al., 2004). Butt et al. (2005), studying immunohistochemically identified synantocytes, showed that they too respond to glutamate application. It is possible that they, existing in early postnatal optic nerves as a fraction of the OPCs, make up a portion of the glial cells activated by NAAG, but likely a minority. Only a fraction (<10%) of cultured microglia respond to 1 mM glutamate application (Light et al., 2006). It is unlikely,

therefore, that microglia, the resident macrophages of the CNS, comprise an important portion of the responding cells in this study. No techniques were used to explicitly identify the types of glia responding in this study.

Electrical Stimulation of Rat Optic Nerve May Increase Glial Ca^{2+} via Released Glutamate or NAAG

It has been shown previously that the glial Ca^{2+} increase is inhibited in NG2-glia of P15 rat optic nerve by suramin (ATP receptor blocker) but not NBQX (AMPA/kainate receptor blocker) (Butt et al., 2005). This suggests that the response to electrical stimulation at that developmental stage under those conditions is not mediated through AMPA receptors, but does not discount other glutamate receptor activation, that glutamate could have a direct effect on astrocytes or OPCs at earlier postnatal ages, that detection of a glutamate response may not have been efficient because of active glutamate uptake or AMPA receptor desensitization, or that the action of glutamate may be mediated through ATP. If CTZ and TBOA are really increasing the glial Ca^{2+} response to electrical stimulation, as suggested by the 5 positively responding nerves in my experiments with electrical stimulation, then there is likely a glutamate or NAAG component to it. This would suggest that the conditions of previous experiments were not optimal to detect the involvement of glutamate or NAAG.

Advantages and Limitations of this Study

This study differs from previous studies using calcium imaging of rat optic nerve because of the care taken to keep the nerve in solution at all times and analytical approaches used. When the nerve was constantly bathed in αCSF, the number of cells showing spontaneous Ca^{2+} oscillations increased. This suggests that the nerves used in this study were in better physiological condition than in previous studies, in which nerves may not have

been continuously bathed. A downside to the high number of oscillating cells is that it was difficult to track a single cell's response without extensive and time-consuming analysis. In other studies, analysis was made on single cells and their individual responses. That is a more powerful technique as the qualitative properties, such as whether the response was spiking or sustained, can be examined. However, if a cell showed a Ca^{2+} increase without stimulation, it was excluded in those studies. My study used a whole nerve analysis in which the number of responding cells at a given time were summed regardless of the qualitative aspects of the response. While this approach has the advantage of not excluding naturally oscillating (and perhaps more physiologically relevant than non-oscillating) cells, it does ignore potentially important features of an individual cell's response.

Summary and Conclusions

Rat optic nerve glia can be stimulated by application of NAAG to increase intracellular Ca^{2+}. The mechanism of action likely is via both intact NAAG acting on metabotropic receptors and glutamate formed from it acting on ionotropic receptors. In the rat optic nerve, NAAG may serve as an axon-glia transmitter and a source of bioactive glutamate. Together, NAAG and GCP may be involved in modulating myelination during development of the CNS as they have been proposed to do in the PNS (Berger and Schwab, 1996; Shah et al., 1996; Yao et al., 1997). In peripheral nerve, GCP activity, and therefore potentially NAAG as well, has been suggested to be linked to myelination. Yao et al. (1997) found that lesioned mouse sciatic nerve treated with the GCP inhibitor 2-PMPA had an increased number of myelinated axons and had axons with thicker myelin as compared to a lesioned nerve without inhibitor. This suggests that GCP plays a role in regulating myelin formation. By inhibiting the enzyme, the extent of remyelination after nerve injury was

increased. In dorsal root ganglia-Schwann cell co-culture, Shah et al. (1998) also found that inhibition of GCP enhanced myelination. Additionally, they found that when application of the inhibitor was delayed to 7 days post-lesion there was no affect on myelination. This finding, together with Berger and Schwab's (1996) finding that in development GCP-II activity is elevated for the first eight postnatal days, but is then reduced in myelinating Schwann cells, suggests that regulation of GCP-II hydrolysis of NAAG may alter myelination. Administration of 2-PMPA 7 days post lesion may not affect myelination because at that time myelinating Schwann cells' GCP-II activity has already been reduced or the cells may be less sensitive to NAAG or to glutamate formed from it. By regulating GCP activity the nervous system can modulate the levels of extracellular glutamate without affecting the chemical's release. For a given amount of NAAG, changes in GCP activity could alter both the amount of glutamate produced and the amount of intact NAAG. As in the periphery, GCP-II activity in the optic nerve increases in the first 2 weeks of development and then decreases (Sherman, Dobrin, Lubischer, and Grossfeld, unpublished data). It is possible, therefore, that NAAG and GCP-II also serve as an important signal for myelination in developing rat optic nerve.

In the optic nerve, current data suggest that both ATP and glutamate are involved in extra-synaptic signaling. However, exactly what the interplay of the two may be is not clear. In studies by Butt et al on P15 rat optic nerve, the effect of electrical stimulation was blocked by ATP receptor blockers, but not by AMPA receptor blockers. This can be explained via two likely mechanisms. Either, upon electrical stimulation of the nerve ATP released from axons causes an increase in glial Ca^{2+}, without glutamate's involvement, or glutamate released from axons acts through non-AMPA glutamate receptors causing ATP release and

subsequent glial Ca^{2+} increase. The fact that ATP receptor blockers blocked the effect of bath-applied glutamate (Butt et al., 2005), supports the second of the two possibilities- that glutamate may stimulate ATP release, which then acts on glia to increase intracellular Ca^{2+}. Whether this sort of interplay between glutamate and ATP is most relevant to the later stage of optic nerve development when OPCs have differentiated to form myelinating oligodendrocytes and synantocytes or also is true of the earlier postnatal ages that I studied is not known at this time.

As this study shows, NAAG too can cause an increase in glial Ca^{2+}. It is possible that electrical stimulation of the optic nerve causes the release of NAAG, which is not an AMPA receptor agonist but a metabotropic glutamate receptor agonist that could cause the release of ATP, which ultimately could cause the response seen.

Besides its activation of neurons and glia at concentrations considered to be physiological, glutamate at higher concentrations can produce pathological changes in neurons and glia and has been implicated in multiple sclerosis, periventricular leukomalacia, and stroke (Alberdi et al., 2006; Johnston, 2005, Matute et al., 2005). It is also possible that NAAG at mM concentrations that might occur when neurons are damaged may have pathological effects and trigger optic nerve degeneration. Increased extracellular glutamate has been linked to excitotoxic cell death (Lucas and Newhouse, 1957; Haas and Erdo, 1991; Yoshioka et al., 1995). The hydrolysis of NAAG to form glutamate may cause an excessive accumulation of extracellular glutamate and result in an excitotoxic condition.

In this context, it should be noted that NAAG has been linked to a variety of neurological disorders, such as ALS, CNS injury, and schizophrenia (Neale et al., 2005; Slusher et al., 1999). Postmortem studies of patients with ALS showed reduced levels of

NAAG and increased GCP activity in the ventral horn of the spinal cord and motor cortex (Tsai et al., 1991). Inhibition of GCP activity significantly delayed the onset of neurological symptoms in a transgenic mouse model for ALS (Ghadge et al., 2003). Neuron and astrocyte cell death was also reduced in trauma models by GCP inhibition (Zhong et al., 2005). In schizophrenia, inhibition of GCP activity also reduced the stereotypic behavioral symptoms (Olszewski et al., 2004). Together, these data suggest a possible neuroprotective role of NAAG, with decreased levels of intact NAAG (or inhibition of GCP activity to reduce glutamate formation) being correlated with ALS onset, protection from CNS injury, and reduced behavioral symptoms of schizophrenia. At this time, it is not clear whether the response of early postnatal rat optic nerve glia to NAAG represents a physiological or pathophysiological condition.

Literature Cited:

Ahmadian, H.; Nielsen, B.; Osborne, H.B.; Johansen, T.N.; Stensbøl, T.B.; Sløk, F.A.; Sekiyama, N.; Nakanishi, S.; Larsen, P.K. & Madsen, U."(S)-homo-AMPA, a specific agonist at the mGlu6 subtype of metabotropic glutamic acid receptors." J Med Chem 1997 40, 3700-3705.

Alberdi, E.; Gómez, M.V.S.; Torre, I.; Domercq, M.; Samartín, A.P.; Cerdá, F.P. & Matute, C."Activation of kainate receptors sensitizes oligodendrocytes to complement attack." J Neurosci 2006 26 , 3220-3228.

Alford, S. & Williams, T.L."Endogenous activation of glycine and NMDA receptors in lamprey spinal cord during fictive locomotion." J Neurosci 1989 9, 2792-2800.

Anderson, K.J.; Borja, M.A.; Cotman, C.W.; Moffett, J.R.; Namboodiri, M.A. & Neale, J.H."N-acetylaspartylglutamate identified in the rat retinal ganglion cells and their projections in the brain." Brain Res 1987 411, 172-177.

Anderson, C.M. & Swanson, R.A."Astrocyte glutamate transport: review of properties, regulation, and physiological functions." Glia 2000 32, 1-14.

Araque, A.; Parpura, V.; Sanzgiri, R.P. & Haydon, P.G."Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons." Eur J Neurosci

1998a 10, 2129-2142.

Araque, A.; Sanzgiri, R.P.; Parpura, V. & Haydon, P.G."Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons." J Neurosci 1998b 18, 6822-6829.

Araque, A.; Li, N.; Doyle, R.T. & Haydon, P.G."SNARE protein-dependent glutamate release from astrocytes." J Neurosci 2000 20, 666-673.

Barcina, J.M.G. & Matute, C."AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter." Brain Res Mol Brain Res 1998 53, 270-276.

Bell, A.H.C.; Finkbeiner, S.M.; Cooper, M.S. & Smith, S.J."Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling." Science 1990 247, 470-473.

Bell, A.H.C. & Finkbeiner, S.M."Ca2+ waves in astrocytes." Cell Calcium 1991 12, 185-204.

Berger, U.V.; Carter, R.E.; McKee, M. & Coyle, J.T."N-acetylated alpha-linked acidic dipeptidase is expressed by non-myelinating Schwann cells in the peripheral nervous system." J Neurocytol 1995 24, 99-109.

Berger, U.V. & Schwab, M.E."N-acetylated alpha-linked acidic dipeptidase may be involved in axon-Schwann cell signalling." J Neurocytol 1996 25, 499-512.

Bergles, D.E.; Roberts, J.D.; Somogyi, P. & Jahr, C.E."Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus." Nature 2000 405, 187-191.

Bevan, S.; Chiu, S.Y.; Gray, P.T. & Ritchie, J.M."The presence of voltage-gated sodium, potassium and chloride channels in rat cultured astrocytes." Proc R Soc Lond B Biol Sci 1985 225, 299-313.

Bjartmar, C.; Battistuta, J.; Terada, N.; Dupree, E. & Trapp, B.D."N-acetylaspartate is an axon-specific marker of mature white matter in vivo: a biochemical and immunohistochemical study on the rat optic nerve." Ann Neurol 2002 51, 51-58.

Billups, B. & Attwell, D."Modulation of non-vesicular glutamate release by pH." Nature 1996 379, 171-174.

Birnbaumer, L.; Campbell, K.P.; Catterall, W.A.; Harpold, M.M.; Hofmann, F.; Horne, W.A.; Mori, Y.; Schwartz, A.; Snutch, T.P. & Tanabe, T."The naming of voltage-gated calcium channels." Neuron 1994 13, 505-506.

Bischofberger, J. & Schild, D."Glutamate and N-acetylaspartylglutamate block HVA calcium currents in frog olfactory bulb interneurons via an mGluR2/3-like receptor." J Neurophysiol 1996 76, 2089-2092.

Blakely, R.D.; Lavollée, L.O.; Thompson, R.C. & Coyle, J.T."Synaptosomal transport of radiolabel from N-acetyl-aspartyl-[3H]glutamate suggests a mechanism of inactivation of an excitatory neuropeptide." J Neurochem 1986 47, 1013-1019.

Blakely, R.D.; Robinson, M.B.; Thompson, R.C. & Coyle, J.T."Hydrolysis of the brain dipeptide N-acetyl-L-aspartyl-L-glutamate: subcellular and regional distribution, ontogeny, and the effect of lesions on N-acetylated-alpha-linked acidic dipeptidase activity." J Neurochem 1988 50, 1200-1209.

Bolton, S. & Butt, A.M."The optic nerve: a model for axon-glial interactions." J Pharmacol Toxicol Methods 2005 51, 221-233.

Boucsein, C.; Zacharias, R.; Färber, K.; Pavlovic, S.; Hanisch, U. & Kettenmann, H."Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro." Eur J Neurosci 2003 17, 2267-2276.

Bowie, D. & Mayer, M.L."Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block." Neuron 1995 15, 453-462.

Bowman, C.L. & Kimelberg, H.K."Excitatory amino acids directly depolarize rat brain astrocytes in primary culture." Nature 1984 311, 656-659.

Brabet, I.; Parmentier, M.L.; Colle, C.D.; Bockaert, J.; Acher, F. & Pin, J.P."Comparative effect of L-CCG-I, DCG-IV and gamma-carboxy-L-glutamate on all cloned metabotropic glutamate receptor subtypes." Neuropharmacology 1998 37, 1043-1051.

Brasnjo, G. & Otis, T.S."Neuronal glutamate transporters control activation of postsynaptic metabotropic glutamate receptors and influence cerebellar long-term depression." Neuron 2001 31, 607-616.

Brovia, V.; Costa, A.; Bedo, G.; Peluffo, H.; Manta, E. & Barbeito, L."N-acetylaspartylglutamate acetoxymethyl triester (NAAG.AM) as a tool for loading the neuropeptides NAAG and succinimidyl-NAAG into intact cells: effect on [3H]-dopamine exocytosis." Braz J Med Biol Res 1996 29, 249-258.

Brew, H. & Attwell, D."Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells." Nature 1987 327, 707-709.

Butt, A.M. & Ransom, B.R."Visualization of oligodendrocytes and astrocytes in the intact rat optic nerve by intracellular injection of lucifer yellow and horseradish peroxidase." Glia 1989 2, 470-475.

Butt, A.M. & Ransom, B.R."Morphology of astrocytes and oligodendrocytes during development in the intact rat optic nerve." J Comp Neurol 1993 338, 141-158

Butt, A.M.; Colquhoun, K.; Tutton, M. & Berry, M."Three-dimensional morphology of

astrocytes and oligodendrocytes in the intact mouse optic nerve." J Neurocytol 1994 23, 469-485.

Butt, A.M. & Jennings, J."Response of astrocytes to gamma-aminobutyric acid in the neonatal rat optic nerve." Neurosci Lett 1994 168, 53-56.

Butt, A.M.; Duncan, A.; Hornby, M.F.; Kirvell, S.L.; Hunter, A.; Levine, J.M. & Berry, M."Cells expressing the NG2 antigen contact nodes of Ranvier in adult CNS white matter." Glia 1999 26, 84-91.

Butt, A.M. & Tutton, M."Response of oligodendrocytes to glutamate and gamma-aminobutyric acid in the intact mouse optic nerve." Neurosci Lett 1992 146, 108-110.

Butt, A.M.; Pugh, M.; Hubbard, P. & James, G."Functions of optic nerve glia: axoglial signalling in physiology and pathology." Eye 2004 18, 1110-1121.

Butt, A.M.; Hamilton, N.; Hubbard, P.; Pugh, M. & Ibrahim, M."Synantocytes: the fifth element." J Anat 2005 207, 695-706.

Bzdega, T.; Crowe, S.L.; Ramadan, E.R.; Sciarretta, K.H.; Olszewski, R.T.; Ojeifo, O.A.; Rafalski, V.A.; Wroblewska B.; Neale J.H.”The cloning and characterization of a second brain enzyme with NAAG peptidase activity.” J Neurochem 2004 89, 627-635.

Cangro, C.B.; Namboodiri, M.A.; Sklar, L.A.; Murphy, A.C. & Neale, J.H."Immunohistochemistry and biosynthesis of N-acetylaspartylglutamate in spinal sensory ganglia." J Neurochem 1987 49, 1579-1588.

Cassidy, M. & Neale, J.H."Localization and transport of N-acetylaspartylglutamate in cells of whole murine brain in primary culture." J Neurochem 1993a 60, 1631-1638.

Cassidy, M. & Neale, J.H."N-acetylaspartylglutamate catabolism is achieved by an enzyme on the cell surface of neurons and glia." Neuropeptides 1993b 24, 271-278.

Charles, A.C.; Merrill, J.E.; Dirksen, E.R. & Sanderson, M.J."Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate." Neuron 1991 6, 983-992.

Charles, A.C.; Naus, C.C.; Zhu, D.; Kidder, G.M.; Dirksen, E.R. & Sanderson, M.J."Intercellular calcium signaling via gap junctions in glioma cells." J Cell Biol 1992 118, 195-201.

Chiu, S.Y. & Kriegler, S."Neurotransmitter-mediated signaling between axons and glial cells." Glia 1994 11, 191-200.

Cirone, J.; Sharp, C.; Jeffery, G. & Salt, T.E."Distribution of metabotropic glutamate receptors in the superior colliculus of the adult rat, ferret and cat." Neuroscience 2002 109,

779-786.

Colomar, A. & Robitaille, R."Glial modulation of synaptic transmission at the neuromuscular junction." Glia 2004 47, 284-289.

Colomé, A.M.L. & de Vivar, M.R."Excitatory amino acid receptors in primary cultures of glial cells from the retina." Glia 1991 4, 431-439.

Cunningham, E.T. & Sawchenko, P.E."Central neural control of esophageal motility: a review." Dysphagia 1990 5, 35-51.

Coco, S.; Calegari, F.; Pravettoni, E.; Pozzi, D.; Taverna, E.; Rosa, P.; Matteoli, M. & Verderio, C."Storage and release of ATP from astrocytes in culture." J Biol Chem 2003 278, 1354-1362.

Coyle, J.T."The nagging question of the function of N-acetylaspartylglutamate." Neurobiol Dis 1997 4, 231-238.

Cull-Candy, S.G.C.; Howe, J.R. & Ogden, D.C."Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurones." J Physiol 1988 400, 189-222.

Curatelo, A.; Arcangelo, P.D.; Lino, A. & Brancati, A."Distributionof N-ACETYL-ASPARTIC and N-acetyl-aspartyl-glutamic acids in nervous tissue." J Neurochem 1965 12, 339-342.

Dani, J.W.; Chernjavsky, A. & Smith, S.J."Neuronal activity triggers calcium waves in hippocampal astrocyte networks." Neuron 1992 8, 429-440.

DeFeudis, F.V."Effects of electrical stimulation on the efflux of L-glutamate from peripheral nerve in vitro." Exp Neurol 1971 30, 291-296.

Domercq, M.; Gómez, M.V.S.; Areso, P. & Matute, C."Expression of glutamate transporters in rat optic nerve oligodendrocytes." Eur J Neurosci 1999 11, 2226-2236.

Donevan, S.D.; Yamaguchi, S. & Rogawski, M.A."Non-N-methyl-D-aspartate receptor antagonism by 3-N-substituted 2,3-benzodiazepines: relationship to anticonvulsant activity." J Pharmacol Exp Ther 1994 271, 25-29.

Duan, S.; Anderson, C.M.; Keung, E.C.; Chen, Y.; Chen, Y.; Swanson, R.A. “$P2X_7$ receptor-mediated release of excitatory amino acids from astrocytes.” J Neurosci 2003 23, 1320 – 8.

Durand, B.; Raff, M. “A cell-intrinsic timer that operates during oligodendrocyte development.” Bioessays 2000 22, 64 – 71.

Fagg, G.E. & Lane, J.D."The uptake and release of putative amino acid neurotransmitters." Neuroscience 1979 4, 1015-1036.

Färber, K. & Kettenmann, H."Physiology of microglial cells." Brain Res Brain Res Rev 2005 48, 133-143.

Fatatis, A. & Russell, J.T."Spontaneous changes in intracellular calcium concentration in type I astrocytes from rat cerebral cortex in primary culture." Glia 1992 5, 95-104.

Fiacco, T.A.; McCarthy, K.D. “Intracellular astrocyte calcium waves in situ increase the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons.” J Neurosci 2004 24, 722 – 732.

Fiacco, T.A.; McCarthy, K.D. “Astrocyte calcium elevations: Properties, propagation, and effects on brain signaling.” Glia 2006 54, 676 – 690.

Fields, R.D. & Burnstock, G."Purinergic signalling in neuron-glia interactions." Nat Rev Neurosci 2006 7, 423-436.

Forloni, G.; Grzanna, R.; Blakely, R.D. & Coyle, J.T."Co-localization of N-acetyl-aspartyl-glutamate in central cholinergic, noradrenergic, and serotonergic neurons." Synapse 1987 1 , 455-460.

Foster, R.E.; Connors, B.W. & Waxman, S.G."Rat optic nerve: electrophysiological, pharmacological and anatomical studies during development." Brain Res 1982 255, 371-386.

Fraser, D.D.; Donnon, L.A.M. & MacVicar, B.A."Astrocytic GABA receptors." Glia 1994 11, 83-93.

Fuhrman, S.; Palkovits, M.; Cassidy, M. & Neale, J.H."The regional distribution of N-acetylaspartylglutamate (NAAG) and peptidase activity against NAAG in the rat nervous system." J Neurochem 1994 62, 275-281.

Fulton, B.P.; Burne, J.F. & Raff, M.C."Visualization of O-2A progenitor cells in developing and adult rat optic nerve by quisqualate-stimulated cobalt uptake." J Neurosci 1992 12, 4816-4833.

Gafurov, B.; Urazaev, A.K.; Grossfeld, R.M. & Lieberman, E.M."N-acetylaspartylglutamate (NAAG) is the probable mediator of axon-to-glia signaling in the crayfish medial giant nerve fiber." Neuroscience 2001 106, 227-235.

Gay, C.; Divry, P.; Macabeo, V. & Gilly, R."[N-acetylaspartic aciduria. Clinical, biological and physiopathological study]" Arch Fr Pediatr 1991 48, 409-413.

Ghadge G.D.; Slusher B.S.; Bodner A.; Canto M.D.; Wozniak K.; Thomas A.G.; Rojas C.; Tsukamoto T.; Majer P.; Miller R.J.; Monti A.L.; Roos R.P. “Glutamate carboxypeptidase II inhibition protects motor neurons from death in familial amyotrophic lateral sclerosis models.” Proc Natl Acad Sci 2003 100(16), 9554-9.

Gupta, B.B.; Seidel, A.; Spessert, R.; Büttner, W.; Klauke, N.; Spanier, J.; Weber, A.; Ziemer, D. & Vollrath, L."In vitro effects of putative neurotransmitters on synaptic ribbon numbers and N-acetyltransferase activity in the rat pineal gland." J Neural Transm Gen Sect 1992 89, 167-178.

Guthrie, P.B.; Knappenberger, J.; Segal, M.; Bennett, M.V.; Charles, A.C. & Kater, S.B."ATP released from astrocytes mediates glial calcium waves." J Neurosci 1999 19, 520-528.

Haas, J. & Erdö, S.L."Quisqualate-induced excitotoxic death of glial cells: transient vulnerability of cultured astrocytes." Glia 1991 4, 111-114.

Hassinger, T.D.; Atkinson, P.B.; Strecker, G.J.; Whalen, L.R.; Dudek, F.E.; Kossel, A.H. & Kater, S.B."Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves." J Neurobiol 1995 28, 159-170.

Hassinger, T.D.; Guthrie, P.B.; Atkinson, P.B.; Bennett, M.V. & Kater, S.B."An extracellular signaling component in propagation of astrocytic calcium waves." Proc Natl Acad Sci U S A 1996 93 , 13268-13273.

Hepp, R.; Perraut, M.; Golaz, S.C.; Galli, T.; Aunis, D.; Langley, K. & Grant, N.J."Cultured glial cells express the SNAP-25 analogue SNAP-23." Glia 1999 27, 181-187.

Hirase, H.; Qian, L.; Barthó, P. & Buzsáki, G."Calcium dynamics of cortical astrocytic networks in vivo." PLoS Biol 2004 2, E96.

Innocenti, B.; Parpura, V. & Haydon, P.G."Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes." J Neurosci 2000 20, 1800-1808.

Izumi, Y.; Shimamoto, K.; Benz, A.M.; Hammerman, S.B.; Olney, J.W. & Zorumski, C.F."Glutamate transporters and retinal excitotoxicity." Glia 2002 39, 58-68.

Jahromi, B.S.; Robitaille, R. & Charlton, M.P."Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ." Neuron 1992 8, 1069-1077.

James, G. & Butt, A.M."Adenosine 5' triphosphate evoked mobilization of intracellular calcium in central nervous system white matter of adult mouse optic nerve." Neurosci Lett 1999 268, 53-56

James, G. & Butt, A.M."P2X and P2Y purinoreceptors mediate ATP-evoked calcium signalling in optic nerve glia in situ." Cell Calcium 2001 30, 251-259.

Janáky, R.; Dohovics, R.; Hermann, A.; Oja, S.S. & Saransaari, P."Effects of metabotropic glutamate receptor agonists and antagonists on D-aspartate release from mouse cerebral cortical and striatal slices." Neurochem Res 2001 26 , 1217-1224.

Jeffery, G.; Sharp, C.; Malitschek, B.; Salt, T.E.; Kuhn, R. & Knöpfel, T."Cellular localisation of metabotropic glutamate receptors in the mammalian optic nerve: a mechanism for axon-glia communication." Brain Res 1996 741, 75-81.

Jeftinija, S.D.; Jeftinija, K.V. & Stefanovic, G."Cultured astrocytes express proteins involved in vesicular glutamate release." Brain Res 1997 750, 41-47.

Joëls, M.; Veldhuizen, M.V.; Urban, I.J. & Kloet, E.R.D."N-acetyl-aspartylglutamate: binding sites and excitatory action in the dorsolateral septum of rats." Brain Res 1987 403, 192-197.

Johnston, M.V."Excitotoxicity in perinatal brain injury." Brain Pathol 2005 15, 234 – 240.

Kew, J.N.C. & Kemp, J.A."Ionotropic and metabotropic glutamate receptor structure and pharmacology." Psychopharmacology (Berl) 2005 179, 4-29.

Kim, W.T.; Rioult, M.G. & Bell, A.H.C."Glutamate-induced calcium signaling in astrocytes." Glia 1994 11, 173-184.

Kowalski, M.M.; Cassidy, M.; Namboodiri, M.A. & Neale, J.H."Cellular localization of N-acetylaspartylglutamate in amphibian retina and spinal sensory ganglia." Brain Res 1987 406, 397-401.

Kriegler, S. & Chiu, S.Y."Calcium signaling of glial cells along mammalian axons." J Neurosci 1993 13, 4229-4245.

Kuhn, S.A.; van Landeghem, F.K.H.; Zacharias, R.; Färber, K.; Rappert, A.; Pavlovic, S.; Hoffmann, A.; Nolte, C. & Kettenmann, H."Microglia express GABA(B) receptors to modulate interleukin release." Mol Cell Neurosci 2004 25, 312-322.

Lavollée, L.O.; Blakely, R.D. & Coyle, J.T."Neurochemical and immunocytochemical studies on the distribution of N-acetyl-aspartylglutamate and N-acetyl-aspartate in rat spinal cord and some peripheral nervous tissues." J Neurochem 1987 48, 895-899.

Lee, S.H.; Kim, W.T.; Bell, A.H.C. & Sontheimer, H."Astrocytes exhibit regional specificity in gap-junction coupling." Glia 1994 11, 315-325.

Lieberman, E.M.; Abbott, N.J. & Hassan, S."Evidence that glutamate mediates axon-to-Schwann cell signaling in the squid." Glia 1989 2, 94-102.

Lieberman, E.M. & Sanzenbacher, E."Mechanisms of glutamate activation of axon-to-Schwann cell signaling in the squid." Neuroscience 1992 47, 931-939.

Light, A.R.; Wu, Y.; Hughen, R.W. & Guthrie, P.B."Purinergic receptors activating rapid intracellular Ca increases in microglia." Neuron Glia Biol 2006 2 , 125-138.

Logan, W.J. & Snyder, S.H."Unique high affinity uptake systems for glycine, glutamic and aspartic acids in central nervous tissue of the rat." Nature 1971 234, 297-299.

Longuemare, M.C. & Swanson, R.A."Excitatory amino acid release from astrocytes during energy failure by reversal of sodium-dependent uptake." J Neurosci Res 1995 40, 379-386.

Lu, X.M.; Tang, Z.; Liu, W.; Lin, Q. & Slusher, B.S."N-acetylaspartylglutamate protects against transient focal cerebral ischemia in rats." Eur J Pharmacol 2000 408 , 233-239.

Lucas, D.R. & Newhouse, J.P."The toxic effect of sodium L-glutamate on the inner layers of the retina." AMA Arch Ophthalmol 1957 58, 193-201.

Malchow, R.P.; Qian, H.H. & Ripps, H."gamma-Aminobutyric acid (GABA)-induced currents of skate Muller (glial) cells are mediated by neuronal-like GABAA receptors." Proc Natl Acad Sci U S A 1989 86, 4326-4330.

Maki, R.; Robinson, M.B. & Dichter, M.A."The glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate depresses excitatory synaptic transmission via a presynaptic mechanism in cultured hippocampal neurons." J Neurosci 1994 14, 6754-6762.

Marrero, H.; Astion, M.L.; Coles, J.A. & Orkand, R.K."Facilitation of voltage-gated ion channels in frog neuroglia by nerve impulses." Nature 1989 339, 378-380.

Massa, P.T. & Mugnaini, E."Cell-cell junctional interactions and characteristic plasma membrane features of cultured rat glial cells." Neuroscience 1985 14, 695-709.

Matute, C.; Gómez, M.V.S.; Millán, L.M. & Miledi, R."Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes." Proc Natl Acad Sci U S A 1997 94, 8830-8835.

Matute, C.; Domercq, M.; Sanchez-Gomez, M.V.“Glutamate-mediated glial injury: Mechanisms and clinical importance.” Glia 2005 53, 212 – 224.

Maubecin, V.A.; Hernandez, F.G.; Williams, J.T. & Bockstaele, E.J.V."Functional coupling between neurons and glia." J Neurosci 2000 20, 4091-4098.

McBain, C.J. & Mayer, M.L."N-methyl-D-aspartic acid receptor structure and function." Physiol Rev 1994 74, 723-760.

Meldrum, B.S."Glutamate as a neurotransmitter in the brain: review of physiology and pathology." J Nutr 2000 130, 1007S-1015S.

Melzer, N.; Salazar, D.T. & Fahlke, C."A dynamic switch between inhibitory and excitatory currents in a neuronal glutamate transporter." Proc Natl Acad Sci U S A 2005 102, 19214-19218.

Menniti, F.S.; Chenard, B.L.; Collins, M.B.; Ducat, M.F.; Elliott, M.L.; Ewing, F.E.; Huang, J.I.; Kelly, K.A.; Lazzaro, J.T.; Pagnozzi, M.J.; Weeks, J.L.; Welch, W.M. & White, W.F."Characterization of the binding site for a novel class of noncompetitive alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonists." Mol Pharmacol 2000 58 , 1310-1317.

Metea, M.R.; Newman, E.A. “Glial cells dilate and constrict blood vessels: A mechanism of neurovascular coupling.” J Neurosci 2006 26, 2862-2870.

Micu, I.; Jiang, Q.; Coderre, E.; Ridsdale, A.; Zhang, L.; Woulfe, J.; Yin, X.; Trapp, B.D.; McRory, J.E.; Rehak, R.; Zamponi, G.W.; Wang, W. & Stys, P.K."NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia." Nature 2006 439, 988-992.

Moffett, J.R.; Cassidy, M. & Namboodiri, M.A."Selective distribution of N-acetylaspartylglutamate immunoreactivity in the extrapyramidal system of the rat." Brain Res 1989 494, 255-266.

Moffett, J.R.; Namboodiri, M.A. & Neale, J.H."Enhanced carbodiimide fixation for immunohistochemistry: application to the comparative distributions of N-acetylaspartylglutamate and N-acetylaspartate immunoreactivities in rat brain." J Histochem Cytochem 1993 41, 559-570.

Moffett, J.R.; Palkovits, M.; Namboodiri, A. & Neale, J.H."Comparative distribution of N-acetylaspartylglutamate and GAD67 in the cerebellum and precerebellar nuclei of the rat utilizing enhanced carbodiimide fixation and immunohistochemistry." J Comp Neurol 1994 347 , 598-618.

Moffett, J.R."Reductions in N-acetylaspartylglutamate and the 67 kDa form of glutamic acid decarboxylase immunoreactivities in the visual system of albino and pigmented rats after optic nerve transections." J Comp Neurol 2003 458, 221-239.

Montana, V.; Malarkey, E.B.; Verderio, C.; Matteoli, M.; Parpura, M.”Vesicular transmitter release from astrocytes.” J Neurosci 2006 54, 700 – 715.

More, J.C.A.; Nistico, R.; Dolman, N.P.; Clarke, V.R.J.; Alt, A.J.; Ogden, A.M.; Buelens, F.P.; Troop, H.M.; Kelland, E.E.; Pilato, F.; Bleakman, D.; Bortolotto, Z.A.; Collingridge, G.L. & Jane, D.E."Characterisation of UBP296: a novel, potent and selective kainate receptor antagonist." Neuropharmacology 2004 47, 46-64.

Neale, J.H.; Bzdega, T. & Wroblewska, B."N-Acetylaspartylglutamate: the most abundant peptide neurotransmitter in the mammalian central nervous system." J Neurochem 2000 75, 443-452.

Neale, J.H.; Olszewski, R.T.; Gehl, L.M.; Wroblewska, B. & Bzdega T.”The neurotransmitter N-acetylaspartylglutamate in models of pain, ALS, diabetic neuropathy, CNS injury, and schizophrenia.” Trends Pharmacol Sci 2005 9, 477-84.

Neary, J.T.; van Breemen, C.; Forster, E.; Norenberg, L.O. & Norenberg, M.D."ATP stimulates calcium influx in primary astrocyte cultures." Biochem Biophys Res Commun 1988 157, 1410-1416.

Nett, W.J.; Oloff, S.H. & McCarthy, K.D."Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity." J Neurophysiol 2002 87, 528-537.

Newman, E.A."Calcium increases in retinal glial cells evoked by light-induced neuronal activity." J Neurosci 2005 25, 5502-5510.

Nicoletti, F.; Meek, J.L.; Iadarola, M.J.; Chuang, D.M.; Roth, B.L. & Costa, E."Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus." J Neurochem 1986a 46, 40-46.

Nicoletti, F.; Wroblewski, J.T.; Novelli, A.; Guidotti, A. & Costa, E."Excitatory amino acid signal transduction in cerebellar cell cultures." Funct Neurol 1986a 1, 345-349.

Noda, M.; Nakanishi, H.; Nabekura, J. & Akaike, N."AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia." J Neurosci 2000 20, 251-258.

Oka, A.; Belliveau, M.J.; Rosenberg, P.A. & Volpe, J.J."Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention." J Neurosci 1993 13, 1441-1453.

Osborne, H.B. & Larsen, P.K."Pharmacology of (S)-homoquisqualic acid and (S)-2-amino-5-phosphonopentanoic acid [(S)-AP5] at cloned metabotropic glutamate receptors." Br J Pharmacol 1998 123, 269-274.

Olszewski R.T.; Bukhari N.; Zhou J.; Kozikowski A.P.; Wroblewski J.T.; Shamimi-Noori S.; Wroblewska B.; Bzdega T.; Vicini S.; Barton F.B.; Neale J.H. “NAAG peptidase inhibition reduces locomotor activity and some stereotypes in the PCP model of schizophrenia via group II mGluR.” J. Neurochem. 2004 89(4), 876-85.

Parpura, V.; Basarsky, T.A.; Liu, F.; Jeftinija, K.; Jeftinija, S. & Haydon, P.G."Glutamate-mediated astrocyte-neuron signalling." Nature 1994 369, 744-747.

Parpura, V.; Fang, Y.; Basarsky, T.; Jahn, R. & Haydon, P.G."Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes." FEBS Lett 1995 377, 489-492

Parpura, V. & Haydon, P.G."Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons." Proc Natl Acad Sci U S A 2000 97, 8629-8634.

Parri, H.R.; Gould, T.M. & Crunelli, V."Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation." Nat Neurosci 2001 4, 803-812.

Parri, H.R. & Crunelli, V."The role of Ca2+ in the generation of spontaneous astrocytic Ca2+ oscillations." Neuroscience 2003 120, 979-992.

Pascual, O.; Casper, K.B.; Kubera, C.; Zhang, J.; Revilla-Sanchez, R.; Sul, J.; Takano, H.; Moss, S.J.; McCarthy, K.; Haydon, P.G. "Astrocytic purinergic signaling coordinates synaptic nerworks." Science 2005 310, 113 – 116.

Pearce, B.; Albrecht, J.; Morrow, C. & Murphy, S."Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux." Neurosci Lett 1986 72, 335-340.

Phillis, J.W.; Song, D. & O'Regan, M.H."Tamoxifen, a chloride channel blocker, reduces glutamate and aspartate release from the ischemic cerebral cortex." Brain Res 1998 780, 352-355.

Pin, J.P.; Colle, C.D.; Bessis, A.S. & Acher, F."New perspectives for the development of selective metabotropic glutamate receptor ligands." Eur J Pharmacol 1999 375, 277-294.

Pinto, J.T.; Suffoletto, B.P.; Berzin, T.M.; Qiao, C.H.; Lin, S.; Tong, W.P.; May, F.; Mukherjee, B. & Heston, W.D."Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells." Clin Cancer Res 1996 2, 1445-1451.

Pitt, D.; Werner, P. & Raine, C.S."Glutamate excitotoxicity in a model of multiple sclerosis." Nat Med 2000 6, 67-70.

Porter, J.T. & McCarthy, K.D."Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals." J Neurosci 1996 16, 5073-5081.

Porter, J.T. & McCarthy, K.D. "Astrocytic neurotransmitter receptors in situ and in vivo." Prog Neurobiol 1997 51, 439 – 455.

Prieto, J.S.; Budd, D.C.; Herrero, I.; Vázquez, E. & Nicholls, D.G."Presynaptic receptors and the control of glutamate exocytosis." Trends Neurosci 1996 19, 235-239.

Prinz, M.; Häusler, K.G.; Kettenmann, H. & Hanisch, U."beta-adrenergic receptor stimulation selectively inhibits IL-12p40 release in microglia." Brain Res 2001 899, 264-270.

Purves, D.; Augustine, G; Fitzpatrick, D.; Katz, L; LaMantia, A.; McNamara J.; William S. *Neuroscience* 2001 2nd Edition. Sinauer Associates, Inc., Sunderland, Massaxhusetts.

Puttfarcken, P.S.; Handen, J.S.; Montgomery, D.T.; Coyle, J.T. & Werling, L.L."N-acetyl-aspartylglutamate modulation of N-methyl-D-aspartate-stimulated [3H]norepinephrine release from rat hippocampal slices." J Pharmacol Exp Ther 1993 266, 796-803.

Ransom, B.R.; Butt, A.M. & Black, J.A."Ultrastructural identification of HRP-injected oligodendrocytes in the intact rat optic nerve." Glia 1991 4, 37-45.

Ransom, B.R. & Orkand, R.K."Glial-neuronal interactions in non-synaptic areas of the brain: studies in the optic nerve." Trends Neurosci 1996 19, 352-358.

Robinson, M.B.; Blakely, R.D. & Coyle, J.T."Quisqualate selectively inhibits a brain peptidase which cleaves N-acetyl-L-aspartyl-L-glutamate in vitro." Eur J Pharmacol 1986 130, 345-347.

Robinson, M.B.; Blakely, R.D.; Couto, R. & Coyle, J.T."Hydrolysis of the brain dipeptide N-acetyl-L-aspartyl-L-glutamate. Identification and characterization of a novel N-acetylated alpha-linked acidic dipeptidase activity from rat brain." J Biol Chem 1987 262, 14498-14506.

Rosenberg, P.A. & Aizenman, E."Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex." Neurosci Lett 1989 103, 162-168.

Rosenberg, P.A.; Amin, S. & Leitner, M."Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture." J Neurosci 1992 12, 56-61.

Rosenmund, C. & Mansour, M."Ion channels: how to be desensitized." Nature 2002 417, 238-239.

Rossi, D.J.; Oshima, T. & Attwell, D."Glutamate release in severe brain ischaemia is mainly by reversed uptake." Nature 2000 403, 316-321.

Rothstein, J.D.; Hoberg, M.D.; Pardo, C.A.; Bristol, L.A.; Jin, L.; Kuncl, R.W.; Kanai, Y.; Hediger, M.A.; Wang, Y.; Schielke, J.P. & Welty, D.F."Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate." Neuron 1996 16, 675-686.

Sager, T.N.; Thomsen, C.; Valsborg, J.S.; Laursen, H. & Hansen, A.J."Astroglia contain a specific transport mechanism for N-acetyl-L-aspartate." J Neurochem 1999 73, 807-811.

Schiffer, H.H.; Swanson, G.T. & Heinemann, S.F."Rat GluR7 and a carboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate." Neuron 1997 19, 1141-1146.

Sekiguchi, M.; Okamoto, K. & Sakai, Y."Low-concentration N-acetylaspartylglutamate suppresses the climbing fiber response of Purkinje cells in guinea pig cerebellar slices and the responses to excitatory amino acids of Xenopus laevis oocytes injected with cerebellar mRNA." Brain Res 1989 482, 87-96.

Serval, V.; Barbeito, L.; Pittaluga, A.; Cheramy, A.; Lavielle, S. & Glowinski, J."Competitive inhibition of N-acetylated-alpha-linked acidic dipeptidase activity by N-acetyl-L-aspartyl-beta-linked L-glutamate." J Neurochem 1990 55, 39-46.

Shah, B.; Yao, Y.; Slusher, B.”NAALADase inhibition enhances myelination in dorsal root ganlia-Schwann cell co-cultures.”Trans. Soc. Neurosci. 1998 28, 616.18.

Sheardown, M.J.; Nielsen, E.O.; Hansen, A.J.; Jacobsen, P. & Honoré, T."2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia." Science 1990 247, 571-574.

Simmons, R.M.; Li, D.L.; Hoo, K.H.; Deverill, M.; Ornstein, P.L. & Iyengar, S."Kainate GluR5 receptor subtype mediates the nociceptive response to formalin in the rat." Neuropharmacology 1998 37, 25-36.

Sladeczek, F.; Pin, J.P.; Récasens, M.; Bockaert, J. & Weiss, S."Glutamate stimulates inositol phosphate formation in striatal neurones." Nature 1985 317, 717-719.

Slusher, B.S.; Robinson, M.B.; Tsai, G.; Simmons, M.L.; Richards, S.S. & Coyle, J.T."Rat brain N-acetylated alpha-linked acidic dipeptidase activity. Purification and immunologic characterization." J Biol Chem 1990 265, 21297-21301.

Slusher, B.S.; Tsai, G.; Yoo, G. & Coyle, J.T."Immunocytochemical localization of the N-acetyl-aspartyl-glutamate (NAAG) hydrolyzing enzyme N-acetylated alpha-linked acidic dipeptidase (NAALADase)." J Comp Neurol 1992 315, 217-229.

Slusher, B.S.; Vornov, J.J.; Thomas, A.G.; Hurn, P.D.; Harukuni, I.; Bhardwaj, A.; Traystman, R.J.; Robinson, M.B.; Britton, P.; Lu, X.C.; Tortella, F.C.; Wozniak, K.M.; Yudkoff, M.; Potter, B.M. & Jackson, P.F."Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury." Nat Med 1999 5, 1396-1402.

Steinhäuser, C. & Gallo, V."News on glutamate receptors in glial cells." Trends Neurosci 1996 19, 339-345.

Stevens, B. & Fields, R.D."Response of Schwann cells to action potentials in development." Science 2000 287, 2267-2271.

Stevens, B.; Porta, S.; Haak, L.L.; Gallo, V.; Fields, D.F.”Adenosine:A neuron-glial transmitter promoting myelination in the CNS in response to action potentials.” Neuron, 2002 36, 855 – 868.

Szatkowski, M.; Barbour, B. & Attwell, D."Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake." Nature 1990 348, 443-446.

Tieman, S.B.; Cangro, C.B. & Neale, J.H."N-acetylaspartylglutamate immunoreactivity in neurons of the cat's visual system." Brain Res 1987 420, 188-193.

Tieman, S.B.; Butler, K. & J.H. Neale"N-acetylaspartylglutamate. A neuropeptide in the human visual system." JAMA 1988 259, 2020.

Tieman, S.B.; Neale, J.H. & Tieman, D.G."N-acetylaspartylglutamate immunoreactivity in neurons of the monkey's visual pathway." J Comp Neurol 1991 313, 45-64.

Tong, G. & Jahr, C.E."Block of glutamate transporters potentiates postsynaptic excitation." Neuron 1994 13, 1195-1203.

Tsai, G.; Stauch, B.L.; Vornov, J.J.; Deshpande, J.K. & Coyle, J.T."Selective release of N-acetylaspartylglutamate from rat optic nerve terminals in vivo." Brain Res 1990 518, 313-316.

Tsai G.C.; Stauch-Slusher B.; Sim L.; Hedreen J.C.; Rothstein J.D.; Kuncl R.; Coyle J.T. “Reductions in acidic amino acids and N-acetylaspartylglutamate in amyotrophic lateral sclerosis CNS.” Brain Res. 1991 556(1), 151-6.

Tsai, G.; Slusher, B.S.; Sim, L. & Coyle, J.T."Immunocytochemical distribution of N-acetylaspartylglutamate in the rat forebrain and glutamatergic pathways." J Chem Neuroanat 1993b 6 , 277-292.

Turecek, R. & Trussell, L.O."Control of synaptic depression by glutamate transporters." J Neurosci 2000 20, 2054-2063.

Urazaev, A.K.; Grossfeld, R.M.; Fletcher, P.L.; Speno, H.; Gafurov, B.S.; Buttram, J.G. & Lieberman, E.M."Synthesis and release of N-acetylaspartylglutamate (NAAG) by crayfish nerve fibers: implications for axon-glia signaling." Neuroscience 2001a 106, 237-247.

Urazaev, A.K.; Gafurov, B.S.; Grossfeld, R.M.; Fletcher, P.L. & Lieberman, E.M."[Synthesis and release of N-acetylaspartyl glutamate (NAAG) in medial giant axons in crayfish]" Ross Fiziol Zh Im I M Sechenova 2001b 87 , 476-491.

Urazaev, A.K.; Grossfeld, R.M. & Lieberman, E.M."Regulation of glutamate carboxypeptidase II hydrolysis of N-acetylaspartylglutamate (NAAG) in crayfish nervous tissue is mediated by glial glutamate and acetylcholine receptors." J Neurochem 2005 93, 605-610.

Valivullah, H.M.; Lancaster, J.; Sweetnam, P.M. & Neale, J.H."Interactions between N-acetylaspartylglutamate and AMPA, kainate, and NMDA binding sites." J Neurochem 1994 63, 1714-1719.

Venance, L.; Stella, N.; Glowinski, J. & Giaume, C."Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes." J Neurosci 1997 17, 1981-1992.

Verderio, C. & Matteoli, M."ATP mediates calcium signaling between astrocytes and microglial cells: modulation by IFN-gamma." J Immunol 2001 166, 6383-6391.

Villegas, J."Axon-Schwann cell interaction in the squid nerve fibre." J Physiol 1972 225,

275-296.

Villegas, J."Effects of tubocurarine and eserine on the axon-Schwann cell relationship in the squid nerve fibre." J Physiol 1973 232, 193-208.

Villegas, J."Cholinergic systems in axon-Schwann cell interactions." Trends in Neurosciences 1978 1, 66.

Villegas, J."Axon/Schwann-cell relationships in the giant nerve fibre of the squid." J Exp Biol 1981 95, 135-151.

Visentin, S.; Renzi, M.; Frank, C.; Greco, A. & Levi, G."Two different ionotropic receptors are activated by ATP in rat microglia." J Physiol 1999 519 Pt 3, 723-736.

Wang, C.; Pralong, W.F.; Schulz, M.F.; Rougon, G.; Aubry, J.M.; Pagliusi, S.; Robert, A.; Kiss, J.Z. “Functional N-methyl-D-aspartate receptors in O-2A glial precursor cells: A critical role in regulating polysialic acid-neural cell adhesion molecule expression and cell migration.” 1996 J Cell Biol 135, 1565 – 1581.

Weinreich, D. & Hammerschlag, R."Nerve impulse-enhanced release of amino acids from non-synaptic regions of peripheral and central nerve trunks of bullfrog." Brain Res 1975 84, 137-142.

Westbrook, G.L.; Mayer, M.L.; Namboodiri, M.A. & Neale, J.H."High concentrations of N-acetylaspartylglutamate (NAAG) selectively activate NMDA receptors on mouse spinal cord neurons in cell culture." J Neurosci 1986 6, 3385-3392.

Wheeler, D.D.; Boyarsky, L.L. & Brooks, W.H."The release of amino acids from nerve during stimulation." J Cell Physiol 1966 67, 141-147.

Williamson, L.C. & Neale, J.H."Calcium-dependent release of N-acetylaspartylglutamate from retinal neurons upon depolarization." Brain Res 1988a 475, 151-155.

Williamson, L.C. & Neale, J.H."Ultrastructural localization of N-acetylaspartylglutamate in synaptic vesicles of retinal neurons." Brain Res 1988b 456, 375-381.

Wong, E.H.; Kemp, J.A.; Priestley, T.; Knight, A.R.; Woodruff, G.N. & Iversen, L.L."The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist." Proc Natl Acad Sci U S A 1986 83, 7104-7108.

Wroblewska, B.; Wroblewski, J.T.; Saab, O.H. & Neale, J.H."N-acetylaspartylglutamate inhibits forskolin-stimulated cyclic AMP levels via a metabotropic glutamate receptor in cultured cerebellar granule cells." J Neurochem 1993 61, 943-948.

Wroblewska, B.; Santi, M.R. & Neale, J.H."N-acetylaspartylglutamate activates cyclic AMP-coupled metabotropic glutamate receptors in cerebellar astrocytes." Glia 1998 24, 172-179.

Yan, H.; Ishihara, K.; Serikawa, T. & Sasa, M."Activation by N-acetyl-L-aspartate of acutely dissociated hippocampal neurons in rats via metabotropic glutamate receptors." Epilepsia 2003 44, 1153-1159.

Yao, Y.; Maulhardt, E.; Slusher, B.”Inhibition of NAALADase activity promotes myelin formation following mouse sciatic nerve cryolesion.”Trans. Soc. Neurosci. 1997 27, 898.

Ye, Z.; Wyeth, M.S.; Baltan-Tekkok, S.; Ransom, B.R. “Functional hemichannels in Astrocytes: A novel mechanism of glutamate release.” J. Neurosci. 2003 9, 3588- 3596.

Yoshioka, A.; Hardy, M.; Younkin, D.P.; Grinspan, J.B.; Stern, J.L. & Pleasure, D."Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage." J Neurochem 1995 64, 2442-2448.

Yuan, X.; Eisen, A.M.; McBain, C.J. & Gallo, V."A role for glutamate and its receptors in the regulation of oligodendrocyte development in cerebellar tissue slices." Development 1998 125 , 2901-2914.

Zhang, Q.; Pangrsic, T.; Kreft, M.; Krzan, M.; Li, N.; Sul, J.; Halassa, M.; Bockstaele, E.V.; Zorec, R. & Haydon, P.G."Fusion-related release of glutamate from astrocytes." J Biol Chem 2004 279, 12724-12733.

Zhao, J.; Ramadan, E.; Cappiello, M.; Wroblewska, B.; Bzdega, T. & Neale, J.H."NAAG inhibits KCl-induced [(3)H]-GABA release via mGluR3, cAMP, PKA and L-type calcium conductance." Eur J Neurosci 2001 13, 340-346.

Zhong, C.; Zhao, X.; Sarva, J.; Kozikowski, A.; Neale, J.H. & Lyeth, B.G."NAAG peptidase inhibitor reduces acute neuronal degeneration and astrocyte damage following lateral fluid percussion TBI in rats." J Neurotrauma 2005 22, 266-276.

Zollinger, M.; Amsler, U.; Do, K.Q.; Streit, P. & Cuénod, M."Release of N-acetylaspartylglutamate on depolarization of rat brain slices." J Neurochem 1988 51, 1919-1923.

Zonta, M.; Angulo, M.C.; Gobbo, S.; Rosengarten, B.; Hossmann, K.; Pozzan, T. & Carmignoto, G."Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation." Nat Neurosci 2003 6, 43-50.

Zur Nieden, R.; Deitmer, J.W. “The role of metabotropic glutamate receptors for the generation of calcium oscillations in rat hippocampal astrocytes in situ.” Cereb Cortex 2006 16, 676 – 687.