Glia as Transmitter Sources and Sensors in Health and Disease

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Short Title: Glia as Transmitter Sources and Sensors

Key Words: glia, glutamate, neurotransmitter, receptor

This work was supported by the National Institutes of Neurological Disorders and Stroke (NS44875 to RF).

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Abstract

Glial cells express a bewildering array of neurotransmitter receptors. To illustrate the complexity of expression, we have assayed non-glutamatergic neurotransmitter receptor mRNA in isolated rat optic nerve, a preparation devoid of neurons and neuronal synapses and from which relatively pure "glial" RNA can be isolated. Of the 44 receptor subunits examined which span the GABA-A, nicotinic, adreno- and glycine receptor families, over three quarters were robustly expressed in this mixed population of white matter glial cells, with several expressed at higher levels than found in control whole brain RNA. In addition to the complexity of glial receptor expression, numerous neurotransmitter release mechanisms have been identified. We have focused on glutamate release from astrocytes, which can occur via at least seven distinct pathways and which is implicated in excitotoxic injury of neurons and glia. Recent findings suggesting that non-glutamatergic receptors can also mediate acute glial injury are also discussed.

Introduction

Glial cells express a multiplicity of neurotransmitter receptors capable of evoking physiological cell-signals, sensitizing the cells to almost any neurotransmitter present in the extracellular space. This is true in particular of astrocytes but numerous functional receptors have also been documented in oligodendroglia and microglia. In a few cases the functional significance of glial receptor expression has been established. For example, the feedback signal mediated by noradrenalin release from neurons to astrocytes which modulates the glutamine-glutamate shunt (see Hertz, 2004). Another example is outlined in the article by Hertz *et al.*, in the current issue. Glia are also richly endowed with neurotransmitter release mechanisms. Taking the example of glutamate and astrocytes, convincing evidence can be marshaled for the presence of at least seven distinct release pathways. Dysfunction of glial neurotransmitter release has clear pathophysiological implications. Loss of astrocyte glutamate homeostasis is a prerequisite for the excitotoxic cascade, a phenomena which is becoming recognized in an increasing number of neurological disorders. Recent evidence has widened the number of neurotransmitters that are implicated in excitotoxic-like injury, and increased the clinical importance of understanding how neurotransmitters are released and sensed in glial cells, and the conditions under which these events become potentially damaging to the nervous system.

Glutamate release:

Astrocytes play the central role in removing glutamate from the extracellular space, an essential component of excitatory signalling in the nervous system. Astrocytes are also replete with glutamate sensors such as ionotropic and metabotropic glutamate receptors (see Gallo & Ghiani, 2000), and are decorated with potential glutamate release mechanisms. While the physiological function(s) of glutamate release and reception in astrocytes is a matter of debate, it is clear that excessive glutamate release is associated with injury in disease states ranging from ischemic lesions such as stroke (see Hazell, 2007) including white matter injury (Karadottir *et al.*, 2005; Salter & Fern, 2005; Micu *et al.*, 2006), through demyelinating disorders like multiple sclerosis (Pitt *et al.*, 2000) to dementias such as Alzheimer's and Huntington's diseases (see Mattson, 2008). The acute injury associated with excess glutamate release is mediated by a well-characterized

excitotoxic cascade involving synaptic exocytosis, depolarization and further synaptic release. Clearly, this pathological cycle requires the failure of astrocyte glutamate regulation, while multiple lines of evidence suggest that astrocytes actively contribute to the feed-forward loop by releasing glutamate during ischemia.

Figure 1 and table 1 near here

There are a plethora of potential glutamate release mechanisms in astrocytes (Fig 1), and experimental evidence can be marshalled to any or all of at least seven distinct pathways of ischemic glutamate release: 1) reverse Na^+ -dependent glutamate uptake, 2) regulatory-volume decrease, 3) hemi-channel opening, 4) necrosis/process loss (clasmotodendrosis), 5) vesicular release, 6) cystine-glutamate exchange and 7) P2X7 channels. Some of these mechanisms appear to be purely pathological, such as necrosis/clasmotodendrosis or hemi-channel formation and are unlikely to mediate glutamate release under physiological conditions. Astrocyte necrosis/clasmotodendrosis will liberate cytoplasmic glutamate, and has been largely ignored as a significant source of ischemic glutamate. However, evidence for early necrosis during ischemia is strong (Fern, 1998, 2001; Thomas *et al.*, 2004; Shannon *et al.*, 2007), while clasmotodendrosis is apparent after a 20 min period of modelled ischemia (Shannon *et al.*, 2007; Salter & Fern, 2008; Fig 2), and is very much region-specific (Shannon *et al.*, 2007). Reverse Na^+ -dependent glutamate uptake and activation of regulatory-volume decrease represent malfunctions of systems that are necessary for proper CNS operation. Vesicular release has been well characterised in reduced systems (see Parpura *et al.*, 2004) and may operate physiologically *in situ* (Bezzi *et al.*, 1998; Fiacco *et al.*, 2007; Shigetomi *et al.*, 2008), but there is currently no evidence for ischemic release via this route. However, all the conditions for vesicular dumping of glutamate from astrocytes exist during ischemia, just as they do for neurons. The significance of astrocyte cystine-glutamate exchange is also unresolved. Located principally in glia (Pow, 2001), net glutamate release in hippocampal slices is only observed at cystine concentrations well above the physiological range (Cavelier & Attwell, 2005). In nucleus accumbens slices, glutamate release is evoked by physiological cystine levels (100-300 nM) (Moran *et al.*, 2005), but

these values are well below the established EC50 for glutamate release via the exchanger (Warr *et al.*, 1999). There is some experimental evidence for ischemic glutamate release via cystine-glutamate exchange in the CNS (Fogal *et al.*, 2007), but the balance of current evidence weights against this being a major glutamate release mechanism.

Figure 2 near here

Oligodendrocytes are also potential sources of ischemic glutamate release, in particular in white matter structures. When examined using immuno-staining at the ultrastructural level, modelled ischemia in neonatal rat optic nerve produces a net increase in glutamate reactivity in oligodendroglia (Wilke *et al.*, 2004). Conversely in periventricular white matter of neonatal rat, hypoxia-ischemia produces a significant depletion of oligodendroglial glutamate indicative of net release (Back *et al.*, 2006), while in cell culture these cells release glutamate that can activate receptors on the host cell and its neighbours resulting in acute injury (Fern & Moller, 2000). The significance of NMDA (Karadottir *et al.*, 2005; Salter & Fern, 2005; Micu *et al.*, 2006) and non-NMDA (McDonald *et al.*, 1998; Fern & Moller, 2000; Follett *et al.*, 2000) glutamate receptors for oligodendroglia injury has been confirmed in a variety of preparations, but the significance of glutamate release from oligodendroglial cells themselves vs., release from astrocytes or axons (Kukley *et al.*, 2007; Ziskin *et al.*, 2007; Alix *et al.*, 2008; Arranz *et al.*, 2008) is not clear.

Other neurotransmitters:

To complicate this picture, glia express numerous non-glutamatergic neurotransmitter receptors. When examined in white matter devoid of neuronal synapses where effects are likely to be glial-mediated, physiological responses can be observed for ATP (Fields & Stevens, 2000; James & Butt, 2002), GABA-A (Simmonds, 1983; Sakatani *et al.*, 1991; Sakatani *et al.*, 1992; Constantinou & Fern, 2009), nicotinic (Zhang *et al.*, 2004; Constantinou & Fern, 2009), adreno- (Honmou & Young, 1995; Saruhashi *et al.*, 1997; Constantinou & Fern, 2009), serotonin (Saruhashi *et al.*, 1997), and glycine (Simmonds, 1983; Constantinou & Fern, 2009) receptors. While there is evidence that

white matter glutamate and ATP receptors help to coordinate the complex morphological arrangement between glia and axons (Ishibashi *et al.*, 2006; Alix *et al.*, 2008), there is little information on the physiological role of other white matter glial receptors and it is not known if their molecular properties differ from receptors in neurons. Despite this, there is growing evidence that white matter glial receptors are important in pathological conditions such as multiple sclerosis, spinal cord injury, stroke, and cerebral palsy (Karadottir *et al.*, 2005; Salter & Fern, 2005; Micu *et al.*, 2006; Alix & Fern, 2009; Constantinou & Fern, 2009; Nikolaeva *et al.*, 2009; Ouardouz *et al.*, 2009a; Ouardouz *et al.*, 2009b).

To illustrate the variety of glial receptor expression, neurotransmitter subunit mRNA was examined in the perinatal (P0) neonatal (P10) and adult (<P45) isolated rat optic nerve (Fig 3). mRNA expression examined in this preparation is almost exclusively of glial origin (Salter & Fern, 2005). Lister-hooded rats were sacrificed in accordance with the regulations of the British Home Office and optic nerves were excised and placed in Phosphate-Buffered Saline. RNA extraction, reverse-transcription and real-time PCR was performed as previously described (Salter and Fern 2005). Primers were designed to amplify short regions of each gene (80-200 bps). Reaction products were loaded on a 1% agarose gel and visualized using SYBR Safe dye (Invitrogen). We considered a product to be present if it had a single band in agarose gel, a mean C(t) value (threshold cycle, n=3) of <30 PCR cycles, and a single peak in the PCR melt curve. To create full-length cDNAs, reverse transcription was performed from either optic nerve or whole brain RNA using PowerScript (Clontech) and mRNA was primed with an oligo(dT)₂₄V primer (SigmaGenosys). Amplification PCR was performed with PfuUltra II Fusion HS DNA Polymerase (Stratagene). To clone PCR products, bands were extracted from agarose gels, purified (Gel Extraction kit; Qiagen), inserted into vector (pGEM-T easy; Promega), and used to transform *E. coli* (XL1-gold ultra-competent cells; Stratagene). DNA insertion was confirmed by PCR screening and positive clones were sequenced.

White matter receptor mRNA expression was highly heterogeneous, with ~80% of the 44 subunits tested detectable in white matter (See Tables 2-5). Levels of mRNA were often high, with ~40% of the tested subunits expressed at a similar level in white matter and whole brain in at least one developmental point, assessed via threshold values

from real-time PCR (glycine receptor $\alpha 4$ and β ; adrenoreceptor $\alpha 2b$, $\alpha 2c$, $\beta 2$ and $\beta 3$; nicotinic cholinergic $\alpha 4$, $\alpha 5$, $\beta 2$, $\beta 3$ and $\beta 4$; and GABA-A $\beta 1$, $\beta 3$, $\gamma 1$, θ , π and $\rho 2$, each repeated three times). These subunit mRNAs were sequenced but no novel splice variants were found and the role these receptors play in white matter glial physiology remains unclear.

Tables 2-5 near here

Functional effects of white matter receptor activation are widely described, for example glycine receptors can modulate action potential conduction in white matter (Simmonds, 1983; Constantinou & Fern, 2009). This is consistent with the expression of $\alpha 2/\alpha 4/\beta$ glycine receptors subunits mRNA reported here, although expression of $\alpha 4$ mRNA is novel. $\alpha 4$ was found in white matter and whole brain at all developmental stages, and was the most enriched of the white matter α subunits. The presence of $\alpha 4$ could explain the unusual pharmacological properties of glycine receptors described in oligodendrocytes (Belachew *et al.*, 1998), which are particularly enriched in white matter.

Figure 3 near here

Functional white matter α and $\beta 1$ adrenoreceptors have also been described (Sanders *et al.*, 2005; Venugopalan *et al.*, 2006; Nikolaeva *et al.*, 2009; Honmou & Young, 1995), and $\alpha 2$ receptor protein is expressed in white matter astrocytes (Nikolaeva *et al.*, 2009). $\beta 1$ receptor activation can affect axon excitability in neonatal white matter but not adult, consistent with the transient $\beta 1$ mRNA expression found here. Extensive $\beta 2$ receptor expression has been reported in human white matter (Dawidek & Robinson, 1993), and our data show this is the major white matter β receptor mRNA present at all ages in white matter. GABA-A receptors can be activated by endogenous GABA in neonatal white matter (Sakatani *et al.*, 1992; Sakatani *et al.*, 1994; Fern *et al.*, 1995; Constantinou & Fern, 2009), and a large number of GABA-A subunit mRNAs were

detected with particularly high relative expression of $\beta 1$, $\beta 3$ and $\gamma 1$. These three subunits are abundant in cultured astrocytes (Bovolin *et al.*, 1992), while oligodendroglia in the hippocampus (Lin & Bergles, 2004), spinal cord astrocytes (Pastor *et al.*, 1995), and unidentified glia in corpus callosum (Bernstein *et al.*, 1996) all express functional GABA-A receptors. The significance of these receptors is not clear, but vesicular neuronal-glial signalling via GABA has been reported in the hippocampus (Paukert & Bergles, 2006). Nicotinic $\alpha 4$, $\alpha 6$, $\beta 2$, and $\beta 3$ subunit protein is present in mature white matter, with lower levels of $\alpha 2$, $\alpha 5$, and $\alpha 7$ (Gotti *et al.*, 2005; Cox *et al.*, 2008), while functional receptors are present in the neonate (Zhang *et al.*, 2004; Guo *et al.*, 2007; Constantinou & Fern, 2009). The current approach showed wider mRNA expression in white matter than previously thought, with $\alpha 2c$, $\alpha 1b$, $\alpha 1d$, $\alpha 2b$, $\beta 1$, $\beta 2$ and $\beta 3$ mRNA found at various developmental points, an expression profile that matches that of oligodendroglia (Rogers *et al.*, 2001).

The expression of non-glutamatergic neurotransmitter receptor mRNA in white matter glia indicated by the current results is similar but not identical to that reported in identified grey matter glial cells. For example, while adrenoreceptor $\beta 1$ subunit expression has been observed previously in grey matter astrocytes, little expression of $\beta 2$ mRNA has been reported in any glial cell isolated from predominantly grey matter brain regions (Cahoy *et al.*, 2008; Hertz *et al.*, 2010). This subunit is robustly present in perinatal, neonatal and adult optic nerve, where levels are similar or higher to that seen in whole brain. Such differences in receptor subunit expression between grey and white matter glial cell populations may indicate significant functional divergence in the role that such receptors play in glial physiology in specific brain locations.

The non-glutamatergic white matter glial receptors found here may have clinical significance. For example, metabolic disorders associated with elevated extracellular glycine levels can produce selective white matter damage (Press *et al.*, 1989; de Koning *et al.*, 2000); prenatal human exposure to the adrenoreceptor $\beta 2$ antagonist terbutaline is associated with reactive glial changes in white matter (Zerrate *et al.*, 2007) and adrenoreceptors may have relevance for disease states affecting white matter from autism to cerebral palsy and stroke (Laudenbach *et al.*, 2002; Zerrate *et al.*, 2007; Constantinou & Fern, 2009; Nikolaeva *et al.*, 2009). In addition, early exposure to nicotine is linked to

selective white matter pathology (Froen *et al.*, 2002; Abdel-Rahman *et al.*, 2005; Jacobsen *et al.*, 2007), while changes in β_2 subunit expression is noted in multiple sclerosis (De Keyser *et al.*, 2004), indicating a largely unexplored relevance to disease. It has been recently shown that neurotransmitters other than glutamate can be directly and acutely harmful to glial cells, in particular nicotinic and adreno-receptors in white matter glia (Constantinou & Fern, 2009; Nikolaeva *et al.*, 2009) (Fig 4). The underlying mechanism is currently unknown, but the phenomena is consistent with the expression of these receptors in glia and may have considerable clinical significance.

Figure legends

Figure 1

Danger to neurons! Glutamate release mechanisms located in astrocytes can potentially contribute to excitotoxicity of neighbouring neurons. Astrocytes surround all parts of neurons, contain elevated levels of free glutamate and are replete with functional release mechanisms capable of contributing to injury under conditions favourable to net release.

Figure 2

Astrocyte vulnerability. Live confocal imaging of isolated P10 GFP/GFAP mouse optic nerve (astrocytes express GFP and are visible within the whole mount preparation, oriented left to right). Two time series are arranged sequentially, as indicated by the times on the left (in min). In the control images a number of astrocyte somata can be seen at T=0 in addition to surrounding glial processes. These remain largely unchanged during 150 min of perfusion with artificial cerebrospinal fluid. In the 20 min OGD series the cells apparent at T=0 are exposed to 20 min of oxygen-glucose withdrawal. Astrocyte processes and somata subsequently disappear from the nerve with time, indicating process loss and necrosis. Taken from Slater and Fern (2008), with permission)

Figure 3

Non-glutamatergic receptor mRNA expression in optic nerve. A: Glycine subunit mRNA expression in adult rat optic nerve ("RON", top panel), whole brain ("Brain", second panel down), neonatal (P10) optic nerve (third panel down) and neonatal whole brain (lower panel). B: Adrenoreceptor subunit mRNA expression. C: Nicotinic receptor subunit mRNA expression. D: GABA-A receptor mRNA expression. E: Selected subunit expression in perinatal (P0) optic nerve (top) and whole brain (bottom). F: The relative difference in brain:optic nerve mRNA expression is plotted as the mean C(t) cycle value ratio, and is shown for all the subunits tested in adult (green bars), neonatal (red bars) and perinatal (when tested, blue bars) brain. Note that for various subunits the relative mRNA expression changes dramatically with age, and that some subunits show higher levels of

expression in the nerve than in brain (negative values) in particular during early development.

Figure 4

Neurotransmitters other than glutamate are toxic to glia. A: Compound action potentials recorded from isolated P10 rat optic nerve. Under control conditions (open squares) the action potential is stable over time (representative potentials are shown from the indicated time points). In nerves exposed to 50 μ M nicotine + 100 μ M noradrenalin (filled bar) experienced a significant loss of the compound action potential, which did not recover following wash-out. B: Electron micrograph showing the ultrastructural features of this injury. Two oligodendroglial cells ("Oli") have lost cell membrane integrity. An astrocyte ("Ast") exhibits swollen endoplasmic reticulum throughout the cytoplasm, swollen mitochondria ("mt") and an unusual form of membrane disintegration (e.g., "*"). C: Quantification of glial cell injury, using a numerical scoring system. "n" refers to the number of identified glial cell based on established ultrastructural criteria. Neighbouring pre-myelinated axons (e.g., arrow head) appear normal. Taken with permission from Constantinou and Fern (2009).

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Table 1

Astrocyte glutamate release mechanism	Activated by:	Physiological signalling?	Ischemia?
Regulatory volume decrease	Swelling. A 20-50 mOsm hypo-osmolarity change or a 3 mM $[K^+]_e$ rise is sufficient (Kimelberg <i>et al.</i> , 1990; MacVicar <i>et al.</i> , 2002).	Astrocyte swelling is activated by physiological neuronal activity (e.g., MacVicar <i>et al.</i> , 2002).	Probably. Volume-activated anion channel blockers reduce ischemic glutamate release (e.g., Feustel <i>et al.</i> , 2004), but drug selectivity is a problem (Ye <i>et al.</i> , 2009).
Na^+ -dependent glutamate transport	Depolarization, $[Na^+]_i$ elevation	Indirectly. Glutamate re-uptake via this mechanism shapes post-synaptic responses (Tzingounis & Wadiche, 2007), an effect that can be modified by Eph-receptor signalling (Filosa <i>et al.</i> , 2009)	Probably during prolonged or severe ischemia (Mitani & Tanaka, 2003; Rossi <i>et al.</i> , 2007; Weller <i>et al.</i> , 2008)
Vesicular release	$[Ca^{2+}]_i$ elevation	Recent evidence is mixed (see Fiacco <i>et al.</i> , 2007; Shigetomi <i>et al.</i> , 2008)	No evidence, but possible.
Clasmatodendrosis	Ischemia (Hulse <i>et al.</i> , 2001; Shannon <i>et al.</i> , 2007; Salter	No	Probably. Process loss can occur within 20 min of

	& Fern, 2008)		ischemia and will liberate intracellular glutamate (Salter et al 2005; Sherwin et al 2007).
Hemi-channels	Low extracellular divalent ion (Ye <i>et al.</i> , 2003).	Not known.	Possible. Ischemia may promote connexin dephosphorylation and hemi-channel opening (Contreras <i>et al.</i> , 2002; Ye <i>et al.</i> , 2003) but see (Suadicani <i>et al.</i> , 2006)
P2X7 receptors	>100 μ M ATP (Duan <i>et al.</i> , 2003)	Probably not. Astrocyte P2X7 expression is low in vivo (Verkhratsky <i>et al.</i> , 2009)	Possibly. Astrocyte P2X7 expression is elevated after ischemia (Franke <i>et al.</i> , 2004).
Cystine-glutamate exchange	Extracellular cystine.	Controversial (Cavelier & Attwell, 2005; Moran <i>et al.</i> , 2005)	Possibly in some injury models (e.g. (Fogal <i>et al.</i> , 2007)

Table 2. Expression levels of glycine receptor subunit mRNA transcripts.

GlyR	P0	P11	Adult
$\alpha 1$	-	-	-
$\alpha 2$	+	+	-
$\alpha 3$	-	-	-
$\alpha 4$	+	+	+
β	++	++	++

Subunit mRNA transcripts that are strongly present are marked “++”, transcripts that are present are marked “+” and subunits that are absent are marked “-”.

Table 3. Expression level of adrenergic receptor subtype mRNA transcripts.

AR	P0	P11	Adult
$\alpha 1a$		-	-
$\alpha 1b$		+	+
$\alpha 1d$		+	+
$\alpha 2a$		-	+
$\alpha 2b$		+	+
$\alpha 2c$	++	++	++
$\beta 1$		+	-
$\beta 2$	++	++	++
$\beta 3$		+	+

Subtype mRNA transcripts that are strongly present are marked “++”, transcripts that are present are marked “+”, and subunits that are absent are marked “-”. Instances where subtypes were not tested are left as blank cells.

Table 4. Expression level of nAChR subunit mRNA transcripts.

nAChR	P0	P11	Adult
$\alpha 2$		-	-
$\alpha 3$		+	+
$\alpha 4$	++	++	+
$\alpha 5$		+	-
$\alpha 6$		-	+
$\alpha 7$		+	-
$\alpha 9$		-	-
$\alpha 10$		-	-
$\beta 2$	+	++	+
$\beta 3$		+	++
$\beta 4$		+	+

Subunit mRNA transcripts that are strongly present are marked “++”, transcripts that are present are marked “+”, and subunits that are absent are marked “-”. Instances where subunits were not tested are left as blank cells.

Table 5. Expression levels of GABA-A R subunit mRNA transcripts.

GABA-A R	P0	P11	Adult
$\alpha 1$		+	+
$\alpha 2$		+	+
$\alpha 3$		+	+
$\alpha 4$		+	-
$\alpha 5$		+	+
$\alpha 6$		+	+
$\beta 1$	++	++	+
$\beta 2$		+	-
$\beta 3$	++	++	++
$\gamma 1$	++	++	++
$\gamma 2$		+	-
$\gamma 3$		+	+
ε		-	-
δ		+	-
θ		-	+
π		+	+
$\rho 1$		+	-
$\rho 2$		+	+
$\rho 3$		-	+

Subunit mRNA transcripts that are strongly present are marked “++”, transcripts that are present are marked “+” and subunits that are absent are marked “-”. Instances where subunits were not tested are left as blank cells.

Figure 1
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Figure 2
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Figure 4
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