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Glutamate and the Biology of Gliomas

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Abstract

Several important and previously unrecognized roles for the neurotransmitter glutamate in the biology of primary brain tumors have recently been elucidated. Glutamate is produced and released from glioma cells via the system x_c^- cystine glutamate transporter as a byproduct of glutathione synthesis. Glutamate appears to play a central role in the malignant phenotype of glioma via multiple mechanisms. By binding to peritumoral neuronal glutamate receptors, glutamate is responsible for seizure induction and similarly causes excitotoxicity which aids the expansion of tumor cells into the space vacated by destroyed tissue. Glutamate also activates ionotropic and metabotropic glutamate receptors on glioma cells in a paracrine and autocrine manner. AMPA glutamate receptors lack the GluR2 subunit rendering them Ca^{2+} permeable and capable of activating the AKT and MAPK pathways. Furthermore, these receptors are critical in aiding the invasion of glioma cells into normal brain. AMPA-Rs accumulate at focal adhesion sites where they may indirectly mediate interactions between the ECM and integrins. Glutamate receptor stimulation results in activation of focal adhesion kinase (FAK) which is critical to the regulation of growth factor and integrin-stimulated cell motility and invasion. The multitude of effects of glutamate on glioma biology supports the rationale for pharmacological targeting of glutamate receptors and transporters. Several ongoing and recently completed clinical trials are exploring the therapeutic potential of interrupting glutamate-mediated brain tumor growth.

Introduction

The last decade has witnessed a significant improvement in our understanding of the mechanisms by which glioma cells manipulate their cellular physiology and local tumor microenvironment to impact a growth advantage. Malignant gliomas arise in a unique environment where abundant vasculature, nutrients and growth factors facilitate tumor proliferation and architectural scaffolding such as white matter tracts provide passage for invading cells to migrate to distant sites throughout the brain. In the context of tumor biology, glioblastoma are similar to other cancers whose malignant behavior is driven by aberrant cell signaling through activated tyrosine kinases and the loss tumor suppressor proteins(Ohgaki, H. and Kleihues, P., 2009). However, targeting classic growth factor pathways in glioma has had limited success in the clinic. Clinical trials using agents that target EGFR, PDGFR, mTOR, PKC and other signaling nodes have been overwhelmingly negative(Adamson, C. *et. al.*, 2009). The implication being that glioma are unique tumors with unrecognized characteristics that are equally or more important. We believe that the neurotransmitter glutamate (Glu), being a highly abundant growth factor and motogen in the brain, is a very good candidate for this role. Glioblastoma tumors release Glu to enhance their highly malignant behavior, and that Glu release via system x_c^- and the excess extracellular Glu this system imparts a survival advantage by promoting resistance to apoptosis and by promoting glioma proliferation and invasion. In fact, the invasive nature of

gliomas enhanced by Glu release is one of the most important limitations to effective disease control; experience demonstrates that more than 80% of glioblastoma recurrences occur within 2-3 cm of the original resection cavity. Successful treatment of malignant gliomas requires recognition of Glu and its receptors as potential targets and novel approaches modulating their influence are needed to improve upon existing ineffective therapies.

Glutamate dynamics in Brain

Glutamate is the main excitatory neurotransmitter in the central nervous system. It is released through Ca^{2+} dependent fusion of synaptic vesicles from activated neurons. Once released, Glu can bind to a number of receptors systems that mediate different biological responses. Fast synaptic transmission is mediated by -amino-3-hydroxy-5-methyl-4-isoaxazolepropionate acid (AMPA-R), while N-methyl-D-aspartate receptor (NMDA-R) activation is typically implicated in coincidence detection and associative learning. In addition, both neurons and glial cells express metabotrobic glutamate receptors (Glu-R) that couple to various second messenger pathways.

Following binding to synaptic Glu-R, Glu is either transported back into presynaptic neuronal terminals or taken up into nearby glial cells. Glial Glu uptake appears to be the predominant route for the degradation of Glu from the synaptic space making it among the most recognized functions of non-malignant astrocytes. Glu clearance occurs primarily via one of two Na^{+}-dependent transporters named EAAT1 or EAAT2, also frequently referred to as Glt-1 and Glast. These transporters are abundant on astrocytic membranes and account for approximately 1% of total brain protein (Danbolt, N.C., 2001). They are localized in close proximity of glutamatergic synapses. In fact, throughout the central nervous system most glutamatergic synapses are tightly ensheeted by astrocytic processes placing their Glu transporters near synaptic release sites. It is generally believed that this arrangement ensures the rapid and effective containment of Glu spillage out of the synaptic cleft. Direct measurements suggest that astrocytic processes in perisynaptic regions experience Glu concentrations as high as ~180 μM (Dzubay, J.A. and Jahr, C.E., 1999) , yet Glu typically does not exceed 1-3 μM elsewhere in the brain. An attractive hypothesis, put forth several decades ago (Schousboe, A. *et. al.*, 1993) suggests the existence of a glial neuronal Glu shuttle whereby Glu is shared between neurons and glia. More specifically, upon uptake into astrocytes, Glu is amidated to glutamine catalyzed by glutamine synthase, a predominantly astrocytic enzyme. Glutamine is released and taken up by nearby neurons where it is hydrolyzed by glutaminase to generate Glu to be packaged into synaptic vesicles.

Release of glutamate from Gliomas

A loss of glial Glu transport has been suggested to contribute to and exacerbate a number of neurological conditions, including stroke, ALS, MS and others (Choi, D.W., 1988). A dysfunction in Glu transport has also been reported in malignant gliomas (Ye, Z.C. and Sontheimer, H., 1999a). The comparison of Glu transport into astrocytes versus their malignant counterparts showed an almost complete absence of Na^{+}-dependent Glu uptake into gliomas. Furthermore Western blots showed a loss of protein expression for EAAT2 and a mislocalization of EAAT1 to the nuclear membrane(Ye, Z.C. *et. al.*, 1999). Even more surprisingly, when the extracellular space was sampled, gliomas appeared to assiduously release significant quantities of Glu into the extracellular space. Within 12h, a monolayer of glioma cells maintained in a culture flask achieved concentrations exceeding 500 μM (Ye, Z.C. and Sontheimer, H., 1999a). This suggests that gliomas behaved exactly opposite to astrocytes, releasing Glu rather than sequestering it. When glioma cells were cocultured with neurons, the released Glu activated neuronal NMDA receptors causing a sustained Ca^{2+} influx resulting in excitotoxic cell death. These findings suggest that a deliberate release of

Glu may promote tumor expansion through the release of excitotoxic concentrations of Glu (Sontheimer, H., 2003) from the tumor as discussed further below.

The initial studies that described the release of Glu and a loss of Glu homeostasis showed the assiduous release of glutamate from cultured human glioma cells(Ye, Z.C. and Sontheimer, H., 1999a) but also demonstrated a complete loss of the Glt-1 and GLAST glutamate transporters in patient biopsy tissues(Ye, Z.C. *et. al.*, 1999). Follow up studies demonstrate Glu release in tumor bearing animals and glioma patients. To accomplish the former, Behrens and colleagues (Behrens, P.F. *et. al.*, 2000) implanted RG2 glioma cells into the cortex of Fisher rats and used microdialysis probes to sample and measure peritumoral Glu. They found that the peritumoral region showed 4-fold elevated Glu compared to either the tumor or the remaining brain. A similar conclusion was drawn by Nedergaard and colleagues (Takano, T. *et. al.*, 2001) who used a bioluminescence detection method to visualize Glu in living brain slices acutely isolated from tumor bearing animals. This study went further and demonstrated that C6 glioma cells that lacked the ability to release Glu failed to grow solid tumors when implanted into rats. Together these studies provided compelling evidence that gliomas release Glu *in vitro, in situ* and *in vivo.*

The issue of whether Glu can be detected in glioma patients remained considerably more controversial and of course more difficult to assess. Most studies that examined this question thus far have used non-invasive imaging techniques such as MRS rather than a direct sampling of the peritumoral extracellular fluid. While some of these studies reported Glu to be elevated in peritumoral brain (Fan, G. *et. al.*, 2004;Rijpkema, M. *et. al.*, 2003), others reported it to be either unchanged or even lower in the tumor (Harris, L.M. *et. al.*, 2008). Fortunately, two studies have placed microdialysis probes into the brain of ambulatory patients allowing a continuous sampling of extracellular Glu (Marcus, H.J. *et. al.*, 2010;Roslin, M. *et. al.*, 2003). Both showed elevated Glu concentrations. One reported Glu concentrations in excess of 100 μM at the tumor margin in all 9 patients examined, yet neither non-malignant brain nor acute brain trauma showed sustained elevation in Glu (Marcus, H.J. *et. al.*, 2010) (Fig. 1). These findings strongly suggest that Glu release has to be considered to be a common attribute of malignant glioma and important in considering the various ways in which Glu affects the tumor biology as elaborated in greater detail below.

Pathway (s) for Glu release

In neurons, the release of Glu occurs exclusively through synaptic vesicles and requires a Ca^{2+} dependent fusion event. By contrasts glial cells have been shown to release Glu in several different ways. Theses include vesicular release (Montana, V. *et. al.*, 2006), reverse operation of the Na^{+}-dependent Glu transporters (Nicholls, D. and Attwell, D., 1990), swelling activated anion channels (Kimelberg, H.K. *et. al.*, 1990) or through hemichannels (Ye, Z.C. *et. al.*, 2003). Biophysical and pharmacological evidence suggests that Glu release from gliomas does not employ these mechanisms but instead occurs predominantly via the system x_c^- cystine Glu exchanger (Ye, Z.C. and Sontheimer, H., 1999a). This abundant amino acid carrier is Na^{+} independent and transports cystine into the cell in exchange for Glu being released. It requires both extracellular cystine and intracellular Glu to function. System x_c^- is a dimer consisting of a catalytic subunit, xCT, and a regulatory protein, CD98, which is required for membrane association of the transporter(Sato, H. *et. al.*, 1999). xCT is a member of the HET class of amino acid transporters characterized by 12 putative transmembrane domains. xCT expression is upregulated in gliomas following oxidative stress(Kim, J.Y. *et. al.*, 2001). The main cellular function of system x_c^- is the uptake of cystine for the generation of the cellular antioxidant glutathione(Chung, W.J. *et. al.*, 2005;Kandil, S. *et. al.*, 2010). As schematized in Fig. 2, the imported cystine reduces to cysteine and assembles with Glu and glycine to form the tripeptide Glu-cysteine-glycine,

glutathione (GSH). GSH is often dubbed the guardian of the cells redox status as it contains highly reactive SH groups that can readily bind to oxidative and nitrosylative radicals. Metabolically active cells, and particularly cancers cells, often synthesize increased amounts of GSH presumably affording them a better protection towards endogenously produced radicals(Estrela, J.M. *et. al.*, 2006). For the system x_c^- transporter to operate, it requires a sustained availability of intracellular Glu and extracellular cystine. Furthermore, several drugs, many of which are analogues to the transported amino acids such as S-4CPG, quisqualate and sulfasalazine inhibit the transporter (Patel, S.A. *et. al.*, 2004). A disruption of system x_c^- expression using shRNA to knock-down the catalytic subunit impairs Glu release *in vivo* (Savaskan, N.E. *et. al.*, 2008). Blockade of cysteine uptake via system x_c^- transport in C6 glioma cells using l-alpohaaminoadipate or l-beta-N-oxalyamino-l-alanine resulted in a greater than 50% reduction in intracellular glutathione levels suggesting that the majority of glioma GSH is due to cysteine uptake through system x_c^-. GSH depletion following x_c^- blockade may promote glioma cell death via activation of the JNK and p38MAPK pathways (Kandil, S. *et. al.*, 2010).

The biochemical source for Glu is not entirely known. It has been shown that omission of glutamine from the culture medium stops GSH synthesis suggesting that Glu may be generated from glutamine via a glutaminase reaction. High levels of liver type glutaminase have been demonstrated in gliomas (Szeliga, M. *et. al.*, 2008). Alternatively, intracellular Glu from glutamine or other sources can be converted to α-ketoglutarate via glutamate-dehydrogenase (GDH) which has been shown to be highly expressed in glioma (Yang, C. *et. al.*, 2009). The oncogene c-myc, highly expressed in treatment resistant glioma stem cells (Wang, J. *et. al.*, 2008), stimulates glutamine metabolism (Wise, D.R. *et. al.*, 2008). Glu can provide carbon intermediates for energy production through the TCA cycle (via α-ketoglutarate) as was recently demonstrated for glutamine via sequential enzymatic activity of GLS and GDH in the setting of glycolysis inhibition (Yang, C. *et. al.*, 2009). Elimination of Glu via multiple pathways is important for cell survival, as discussed below, and can serve to provide alternate sources of energy for glycolysis-impaired cells and protection from free radicals.

Another class of transporters, the excitatory amino acid transporters EAAT1-5 has been implicated in Glu release from neurons and glial cells at least under pathophysiological condition. The predominant glial transporters are EAAT1&2 and are responsible for the Na^+-dependent uptake and clearance of Glu from the extracellular space. Under conditions of energy failure, such as stroke, EAAT transporters can run backwards exporting rather than importing Glu(Allen, N.J. *et. al.*, 2004). However, these transporters do not appear to be functional in most gliomas and are either not expressed or mislocalized(Ye, Z.C. *et. al.*, 1999). Advanced glial tumors lack expression of EAAT-2. We found that while low-grade astroglial tumors have relatively high levels of EAAT-2, high-grade glioblastoma tumors have much lower levels. Glioblastomas had almost no EAAT-2 expression, as shown by immunohistochemistry. Interestingly, the presence of EAAT2 in high grade gliomas appears to be toxic inducing glioma apoptosis in a caspase-dependent manner and an increase in EAAT-2 expression ex vivo significantly reduced glioblastoma cell tumorgenicity(de Groot, J.F. *et. al.*, 2005). Thus, the release of Glu is important for maintaining glioma cell viability on multiple fronts.

Taken together, current evidence suggests that Glu is being released from gliomas as an obligatory byproduct of cellular cystine uptake for the generation of the antioxidant GSH, as illustrated in Fig. 2. The GSH produced in this context has a clear protective function for the tumor. The released Glu has a multitude of targets all of which benefit the tumor yet compromise the patient.

Cellular target of the released Glutamate

Gliomas are surrounded by other glioma cells, as well as neurons, glial, microglial and vascular cells. All of these have been demonstrated to be responsive to Glu. The receptor systems activated and the ensuing signaling events following binding of Glu differ greatly between cells.

Neuronal response to glioma released Glu—As already discussed above, excessive amounts of Glu can cause neuronal excitotoxicity and hence Glu release from gliomas may similarly exert peritumoral excitotoxicity (Fig. 2). This may be one mechanism whereby the tumor vacates room for its expansion. Unlike systemic cancers, which grow in soft tissue, brain tumors have little room to expand since brain tissue cannot be displaced beyond the boundaries of the calvarium. Excitotoxicity is believed to result from the aberrant activation of neuronal NMDA receptors in the peritumoral tissue manifesting as hyperexcitability and may explain the frequent occurrence of seizures emanating from the tumor and surrounding brain. Over 80% of glioma patients suffer seizures during the course of their diseases (Moots, P.L. *et. al.*, 1995) and in many patients seizures are an early sign. The measured Glu release from gliomas is large enough to suggest an overactivation of neuronal Glu receptors. However, it has not been unequivocally demonstrated that seizures in peritumoral neurons are caused by the release of Glu from the tumor. What has been demonstrated, however, is wide spread excitotoxicity (Takano, T. *et. al.*, 2001) as well as cellular edema (Savaskan, N.E. *et. al.*, 2008). Clearly, neurons are lost in the vicinity of the tumor and inhibition of Glu release via system x_c^- prevents this from happening and slows tumor growth in tumor bearing mice (Chung, W.J. *et. al.*, 2005).

Glia—Astrocytes are typically resistant to even millimolar concentrations of Glu, whereas oligodendrocytes do not tolerate prolonged Glu exposure well. Indeed, much like neuronal excitotoxicity, oligodendrocytes rapidly die following prolonged exposure to excessive Glu (Oka, A. *et. al.*, 1993). As it pertains to peritumoral astrocytes, their response to excess Glu is not well understood. Astrocytes typically respond to neuronally released Glu by the rapid uptake into the cytoplasm using the EAAT1 &EAAT2 transporters. It is clear, however, that the Glu release from the tumor must overwhelm the astrocytic capacity to sequester Glu and maintain proper Glu homeostasis. It is possible that astrocytes lose functional Glu transporters or alternatively, lose the ability to catabolize Glu to glutamine. It has been shown that reactive gliosis causes a loss of glutamine synthetase (GS), the enzyme involved in the conversion of Glu to glutamine (Ortinski, P.I. *et. al.*, 2010). If peritumoral astrocytes lose GS, this loss could explain the observed dysregulation of Glu. Such a loss may also contribute to the observed hyperexcitability in tumor associated brain. A recent study (Ortinski, P.I. *et. al.*, 2010) showed that the loss of GS in reactive astrocytes led to reduced GABAergic signaling presumably by a disruption of the glial neuronal Glu glutamine cycle. Ultimately, the space occupied by expanding tumors is devoid of neurons and astrocytes alike. Low-grade glioma contain a lower density of tumor cells intermixed with normal brain and as these tumors progress to higher grade there is a complete loss of normal brain suggesting that astrocytes too must be killed by the expanding tumor. The interaction between glia-glioma cells therefore certainly warrants further study.

Astrocytes also express ionotropic and metabotrobic Glu receptors. AMPA ionotropic receptors are abundantly expressed on astrocytes as functional ion channels. AMPA activation can alter astrocyte morphology, increase the release of Glu from type-2 astrocytes and modulate gene expression which may trigger long-term changes in cellular function (Seifert, G. and Steinhauser, C., 2001). Metabotropic Glu receptor (mGluRs) expression on human astrocytes is dynamic depending on environmental concentrations of growth factors (Aronica, E. *et. al.*, 2003) and can also change in response to injury (Ferraguti, F. *et. al.*,

2001;Ulas, J. *et. al.*, 2000). mGluRs appear to regulate the expression of Glu transporters (Aronica, E. *et. al.*, 2003) and inhibition of mGluR with S-4CPG significantly reduced the release of Glu from astrocytes (Ye, Z.C. and Sontheimer, H., 1999b).

Glioma—Multiple Glu receptor subtypes have been shown to be expressed on glioma cells in culture and in primary glioblastoma specimens. Ionotropic AMPA, kainate and NMDA receptors as well as metabotropic Glu receptors are expressed in multiple solid tumor types including gliomas (de Groot, J.F. *et. al.*, 2008;Ishiuchi, S. *et. al.*, 2007;Stepulak, A. *et. al.*, 2009). Most studies have focused on a single or small number of cell lines, primary cultures or a limited number of patient samples. It appears that the a-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate acid type receptor (AMPA-R) including the subunits GluR1, 3 and 4 are highly expressed in high grade gliomas (de Groot, J.F. *et. al.*, 2008;Ishiuchi, S. *et. al.*, 2007). One report demonstrated kainate receptors on several glioma cell lines in culture (Stepulak, A. *et. al.*, 2009). NMDA subunits NR2A, B, and C and NR3A were shown to be present on several glioma cell lines (Stepulak, A. *et. al.*, 2009). Other reports do not show the expression of specific receptor types but demonstrate an anti-tumor effect with NMDA antagonists (Rzeski, W. *et. al.*, 2001). Finally, several studies have described the expression of metabotropic receptors mGluR3 and mGluR5 in glioma cell lines and both low and high grade glioma specimens (Condorelli, D.F. *et. al.*, 1997;Stepulak, A. *et. al.*, 2009). Unfortunately, a study of the prevalence of the different Glu receptor subtypes in a large number of human glioma samples has not been accomplished. Ideally, clinical studies will incorporate Glu receptor target expression in fresh surgery specimens as a study requirement with the ultimate goal of correlating receptor expression with clinical outcome to Glu-targeted therapy.

AMPA-R activations and glioma invasion

As mentioned above most glioma cells express AMPA receptors. In neurons, these receptors mediate fast excitatory synaptic transmission. They are heteromultimers composed of the GluR1-GluR4 subunits. All gliomas investigated thus far contain at least GluR1 together with either GluR3 or GluR4. The presence of an additional GluR2 subunit critically influences Ca^{2+} permeability. If an unedited from of this subunit is expressed Ca^{2+} permeable receptors are established whereas the edited form of GluR2 renders AMPA-R Ca^{2+} impermeable. This is typically the case at synapses. Studies to date suggest that the majority of gliomas lack GluR2 expression and hence binding of Glu causes the influx of Ca^{2+} through AMPA-R. In a gene expression analysis of several hundred glioblastoma tumor specimens, loss of *GRIA2* (gene for GluR2) expression was one of 38 gene changes that predict a poor prognosis in glioblastoma (Colman, H. *et. al.*, 2010) suggesting that calcium entry via the AMPA receptor is a critical component of glioblastoma biology. The Ca^{2+} influx has been shown to be essential in promoting cell motility and cell invasion. Ishiuchi et al overexpressed the GluR2 subunit in gliomas and the resulting loss of Ca^{2+} permeability was sufficient to render cells unable to invade in vitro and in vivo (Ishiuchi, S. *et. al.*, 2007). While the source of Glu could be neuronal, the above discussed continuous release of Glu allows for a scenario whereby glioma cells may activate AMPA-R on the same or neighboring cells (Fig. 3). Evidence for such autocrine or paracine activation has been demonstrated (Lyons, S.A. *et. al.*, 2007) to result in Ca^{2+} oscillations correlating with cell motility. The inhibition of system x_c^- mediated Glu release disrupted glioma invasion (Lyons, S.A. *et. al.*, 2007). Interestingly, in gradient chambers glioma cells migrated towards elevated Glu concentrations suggesting that Glu could be considered a chemo attractant. Since glioma cells frequently show chain migration, particularly along blood vessels, the leading cell, through release of Glu, may stimulate trailing cells to follow.

Recent studies have elucidated several mechanistic ideas as to how AMPA-R on glioma cells may regulate cell motility and tumor invasiveness. Overexpression of GluR1, the most abundant subunit of the AMPA-R in glioma cells, results in an increase in glioma adhesion to extracellular matrix (ECM) components such as collagen. AMPA-Rs appear to accumulate at focal adhesion sites where they may indirectly mediate interactions between the ECM and integrins. The enhanced adhesion to the ECM is mediated via increased surface expression of β1-integrin and subsequent activation of focal adhesion kinase (FAK). Overexpression of the GluR1 subunit of the AMPA-R in glioma cells activates FAK independent of AMPA-R activation. Although the mechanism through which GluR1 expression can promote FAK activation is unknown, it may be related to FERM protein or integrin engagement. Stimulation of the AMPA-R with Glu or AMPA promotes cellular detachment mediated through Rac1 and causes an increase in transwell migration *in vitro* and an increase in tumor invasion *in vivo* (Piao, Y. *et. al.*, 2009) (see Fig 3). Interestingly, overexpression of the AMPA receptor led to predominantly perivascular and subpial patterns of invasion similar to that observed following prolonged antiangiogenic therapy in a orthotopic glioma model (Piao, Y. *et. al.*, 2009).

Glu receptor activation and growth control

Activation of AMPA and NMDA receptors are important for neuronal migration, survival, and differentiation and may be important for proliferation of neuronal precursor cells (Contestabile, A., 2000;Komuro, H. and Rakic, P., 1993;Nguyen, L. *et. al.*, 2001). AMPA receptors on normal cells in the CNS, in addition to their function as ion channels, activate signal transduction cascades (Schenk, U. *et. al.*, 2005). AMPAR stimulation on normal neurons increases the activation of mitogen activated protein kinase (MAPK) through a G-protein β subunit and Ras/Raf/MEK-1 complex (Wang, Y. and Durkin, J.P., 1995) and via interactions with members of the non-receptor tyrosine kinase family Src (Hayashi, T. *et. al.*, 1999). Calcium entry through NMDA receptors is a potent regulator of the signaling pathways mediated by this receptor. Glutamatergic stimulation of the NMDA receptor can lead to activation of mTOR-S6 kinase, AKT and MAPK pathways (Lenz G and Avruch J, JCB 2005). Ca^{2+} influx through the AMPA-R has been shown to lead to AKT activation (Ishiuchi S et al. J Neurosci 2007). Both NMDA and AMPA receptor signaling are thought to be critical for activating a series of complex pathways responsible for maintaining synaptic strength and the encoding and “survival” of long-term memories (Malinow, R. and Malenka, R.C., 2002;Thomas, G.M. and Huganir, R.L., 2004). Conceptually, it is perhaps not surprising that tumors of glial origin manipulate Glu receptors to promote limitless proliferation and survival via the activation of Glu receptors.

AMPA receptors are highly expressed in glioblastoma and perform critical functions in glioma biology and enhance its malignant phenotype. As mentioned previously, glioblastoma appear to express low levels of the GluR2 subunit which contributes to AMPA-R calcium permeability. Rzeski et al. demonstrated that antagonism of AMPA Glu receptors or the removal of calcium from the culture medium in different tumor types including glioma decreased cell proliferation via both decreased cell division and increased cell death, suggesting that a common tumor proliferation mechanism involves Glu and Glu receptors (Rzeski, W. *et. al.*, 2001). Similarly, overexpression of AMPA-R promoted glioma proliferation in vitro and reintroduction of GluR2 via adenoviral vector blocked this proliferation and induced apoptosis (Ishiuchi, S. *et. al.*, 2002). Thus, glioma regulation of AMPA receptor subunit expression and calcium permeability may represent a novel mechanism for signal transduction pathway activation.

Several mechanisms by which AMAP-Rs are able to modulate cell proliferation and survival have recently been described (see Fig 3). Ishiuchi et al. recently demonstrated that calcium influx through AMPA receptors promotes glioma proliferation through activation of AKT

which was blocked with a dominant negative form of AKT (Ishiuchi, S. *et. al.*, 2007). These data strongly suggest that calcium entry via the AMPA-R is able to activate AKT. Additionally, it was recently demonstrated that AMPA-R stimulation promotes EGFR expression (Schunemann, D.P. *et. al.*, 2010) and insertion of GluR2 decreases EGFR expression (Beretta, F. *et. al.*, 2009). Thus, glutamate could both directly and indirectly activate the PI3K/AKT pathway via calcium entry and by promoting EGFR signaling, respectively. The PI3K/AKT plays a central role in the malignant phenotype of glioma (Knobbe, C.B. *et. al.*, 2002) but its activation has traditionally been thought to be due to overexpression and/or constitutive activation of the highly oncogenic epidermal growth factor receptor (EGFR) or platelet derived growth factor (PDGFR). A positive feedback loop may exist between AMPA-R and EGFR, both of which can activate the PI3K/AKT pathway. The idea that AMPA receptors may mediate AKT activation and EGFR expression completely changes the current thinking within the field and requires a paradigm shift in our approach to blocking this pathway.

The MAPK pathway has also been implicated to be important in promoting tumor cell proliferation (Reddy, K.B. *et. al.*, 2003). There is growing evidence that activation of the MAPK pathway is involved in the pathogenesis, progression, and oncogenic behavior of glioblastoma (Newton, H.B., 2003;Rajasekhar, V.K. *et. al.*, 2003). Current data suggests that activation of the Ras/MAPK signaling pathway predominantly occurs through aberrant expression and over activity of membrane receptor tyrosine kinases including EGFR, PDGFR and IGF-1R (Besson, A. and Yong, V.W., 2001;da Rocha, A.B. *et. al.*, 2002). We recently showed that Glu, acting as a growth factor via AMPAR stimulation, also contributes to MAPK activation in glioma. MAPK is activated in a time and dose-dependent fashion following exposure to AMPA, which could be blocked with an AMPA receptor antagonist or MEK1 inhibitor. Retroviral delivery of GluR1 shRNA in two different cell lines reduced GluR1 protein expression, inhibited AMPA-mediated increases in MAPK phosphorylation, and decreased glioma proliferation *in vitro* (de Groot, J.F. *et. al.*, 2008). Finally, MAPK signaling via calcium influx through AMPA-R may also be mediated in part via SRC (Beretta, F. *et. al.*, 2009), a known signaling mediator of cell proliferation and invasion in glioblastoma (de Groot, J. and Milano, V., 2009). In summary, glioma cells may utilize AMPA-R in an autocrine or paracrine fashion to promote cell proliferation and survival via multiple signaling nodes already known to be important in glioma biology.

NMDA receptor inhibition has also been shown to decrease glioma proliferation. Although less widely studied, several reports have demonstrated the potential for inhibiting tumor growth and proliferation with the use of NMDA antagonists. The NMDA receptor antagonist dizocilpine was able to decrease proliferation of astrocytoma cells in culture (Rzeski, W. *et. al.*, 2001). In lung cancer cell lines, it was later shown that the antiproliferative effects of dizocilpine were related to inhibition of MAPK signaling and a reduction of phosphorylated CEBP, decreased expression of cyclin D1, and up-regulation of p21 and p53 (Stepulak, A. *et. al.*, 2005). Although the NMDA-mediated signaling pathways in glioma are unknown, it is possible that different tumor types have evolved to manipulate Glu receptor signaling to promote malignant growth in similar ways. Importantly, blockade of AMPA and NMDA receptors enhanced the effectiveness of traditional cytotoxic chemotherapy in several cancer cell lines (Rzeski, W. *et. al.*, 2001) underscoring the potential utility of using combinations of different treatment modalities.

Clinical opportunities

The recognition that Glu, a ubiquitous transmitter and growth factor within the extracellular environment, promotes tumor growth and invasion requires serious attention as a potential therapeutic target. If blockade of the AMPA-R receptor can limit tumor invasion, this could transform the field of neuro-oncology since no other therapeutic modality has been shown to

effectively inhibit glioma infiltration in human studies. Similarly if inhibition of Glu release via system x_c^- disrupt the growth promoting and invasive effects of Glu a novel avenue for therapeutic intervention would be opened. Indeed, some early clinical trials are already under way and are briefly summarized here.

Targeting Glu-R with Talampanel—Since there is general consensus in the field that Glu released from gliomas targets Glu-R on adjacent neurons and on glioblastoma tumor cells, inhibitors of Glu receptors appeared a logical point of intervention. Unfortunately, most Glu receptor inhibitors have shown severe side-effects when used clinically in the past (Lipton, S.A., 2004), in part because Glu receptors underlie the majority of normal signaling in the brain and activation of the NMDA receptor underlies learning and memory. A recently developed noncompetitive antagonist of the AMPA receptor named Talampanel showed excellent brain penetration (Calabrese, C. *et. al.*, 2007) and produced favorable data in preclinical studies (Goudar, R.K. *et. al.*, 2004). Two independent clinical trials have been conducted with this drug for the treatment of patients with malignant glioma (Grossman, S.A. *et. al.*, 2009;Iwamoto, F.M. *et. al.*, 2010). In a single arm phase II study, Talampanel was administered to 32 patients diagnosed with either recurrent GBM or anaplastic glioma as mono-therapy. It reported excellent safety of the drug with no adverse side effects. However, the study also showed no significant activity with similar median overall survival times compared to historical controls (Iwamoto, F.M. *et. al.*, 2010). A second, larger phase II study (Grossman, S.A. *et. al.*, 2009) was performed in newly diagnosed glioblastoma patients. Talampanel was given in combination with radiation therapy plus TMZ in a single arm, multi-center consortium study. The primary endpoint of the study was efficacy as measured by overall survival. Radiographic response, progression free survival and safety were also assessed. Data just released from this study which enrolled 72 patients report encouraging survival results and an absence of added toxicity from the talampanel, suggesting a more wide-spread use of this combination therapy (Grossman, S.A. *et. al.*, 2009). However, a randomized placebo-controlled trial will be necessary to more unequivocally assess the efficacy of this treatment regimen compared to radiation and temozolomide alone, now considered standard of care.

Targeting other Glu Receptors: NMDA and mGluRs—Although preclinical data on anti-tumor effects is not as secure as for the AMPA receptor, there is emerging evidence that inhibiting other Glu receptors may provide a clinical benefit to patients with glioma. Memantine, an uncompetitive NMDA receptor channel blocker approved for the treatment of Alzheimer's disease, has been shown in preclinical models to decrease glioma proliferation (Rzeski, W. *et. al.*, 2001) potentially via inhibition of MAPK (Stepulak, A. *et. al.*, 2009). In addition to its antiproliferative effects, memantine may also prevent excitotoxic neuronal cell death due to excess extracellular glutamate. This could have the potential to preserve neurologic function and block the creation of pathways for glioma cells to spread to distant sites (Rothstein, J.D., 2002). The Radiation Therapy Oncology Group (RTOG)-0614 (NCT00566852) trial is a randomized, blinded, placebo controlled phase III trial of memantine to prevent cognitive dysfunction in patients with brain metastasis undergoing radiation therapy. This novel approach should be evaluated in patients with glioblastoma who may benefit from both the neuron-protecting actions of memantine as well as the potential for this agent to block tumor proliferation and excitotoxicity-mediated invasion. Preclinical studies demonstrating that inhibiting NMDA receptors is synergistic with chemotherapy emphasize the need to pursue combination therapy strategies when targeting glutamate receptors.

Similarly, metabotropic Glu receptor inhibition could provide a benefit through multiple mechanisms. Group I mGluRs can potentiate NMDA-mediated excitotoxicity which, as described above, could induce neurologic dysfunction, seizures and enhance tumor invasion.

Inhibition of mGluRs found on glioma cells could also block glioma proliferation as was recently shown for LY341495, a selective Group II mGluR antagonist (Arcella, A. *et. al.*, 2005). Studies are limited by the lack of approved mGluR antagonists and there are currently no mGluR-targeting agents in clinical trials at this time.

Targeting Glu release with sulfasalazine (SAS)—A second approach suggested from preclinical studies would be to target Glu release from gliomas. The system x_c^- transporter appears to be responsible for the majority of Glu release and can be effectively inhibited using sulfasalazine, an FDA approved drug shown to slow tumor growth in mice (Chung, W.J. *et. al.*, 2005;Robe, P.A. *et. al.*, 2004). A phase I study of this agent was recently terminated (Robe, P.A. *et. al.*, 2009). This study enrolled 9 heavily pretreated patients with poor performance (median KPS of 50) who received various doses of SAS. As a phase I study, the primary endpoint was safety although these outcomes are not obvious. The study was terminated by the investigator due to concerns about the treatment's poor efficacy. This study was not adequately designed to assess efficacy and thus their results should not be taken as an indication of SAS inactivity. Given the potential for SAS to control Glu release and potentially block seizures, we recently opened a phase I study to examine SAS in low-grade newly diagnosed glioma patients with the objective to measure Glu release in patients before and after administration of SAS using MRS. The impact of SAS on seizures, a potential surrogate for excitotoxic levels of extracellular Glu, will also be assessed. The advantage of this study design is that a biological marker, namely Glu levels will be indirectly measured using non-invasive imaging techniques. Patient selection criteria require good Karnofsky scores above 90. It is also important to consider that SAS may not be the ideal drug choice. Although FDA approved, SAS has very poor systemic bioavailability due to its rapid cleavage by colonic bacteria into inactive constituents. Once sulfasalazine is in circulation it is rapidly metabolized with a half-life of ~80 minutes. The BBB permeability of SAS is unknown. That said a SAS analogue with better bioavailability and known BBB penetration should be developed in investigated in future clinical studies.

Upregulation of Glu reuptake—The dysregulation of Glu is the consequence of enhanced Glu release from gliomas combined with a near complete loss of reuptake via glial EAAT transporters. Enhancing EAAT mediated Glu reuptake therefore offers another opportunity to intervene. Proof of principle that this approach would stop glioma expansion was provided in cell culture (Guo, H. *et. al.*, 2002) and in preclinical studies (de Groot, J.F. *et. al.*, 2005;Vanhoutte, N. *et. al.*, 2009) where the ectopic expression of recombinant EAAT2 transporters in glioma cells inhibited their growth *in vitro* and tumor growth *in vivo*. However, clinical translation of this concept appears elusive at this point. Interestingly, EAAT2 transporter expression can be enhanced 2-3 fold in mice by giving the beta-lactam antibiotic ceftriaxone (Rothstein, J.D. *et. al.*, 2005). It is therefore possible that administration of such antibiotics could enhance the glial reuptake of Glu via EAAT2 thereby ameliorating the Glu accumulation in peritumoral tissue. This approach could be readily explored in combination with other treatments.

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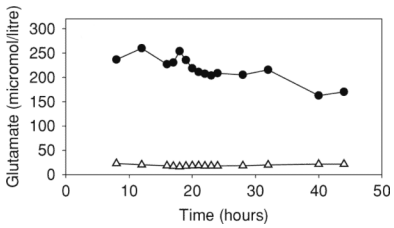

Fig. 1.

Glutamate release measured over 44h by microdialysis in a fully ambulatory 57 year old male patient diagnosed with a grade IV GBM in the immediate tumor vicinity (closed circles) versus 2 cm distant in uninvolved brain (open triangles) with From Marcus et al., J. Neurooncol., 2010:97, p14.

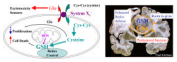

Fig. 2.
Glutamate is released from glioma cells in conjunction with the uptake of cystine for the cellular synthesis of the antioxidant glutathione. It is hypothesized to initiate excitotoxicity in the peritumoral brain.

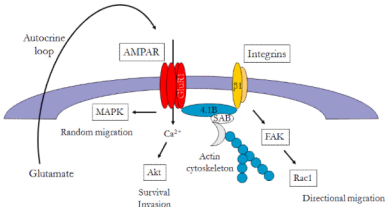

Fig 3.

Autocrine or paracrine AMPA-R activation enhances cell proliferation and survival via calcium mediated activation of PI3K/AKT and MAPK. Receptor stimulation enhances cell motility and invasion via integrin interaction and downstream activation of FAK. From Piao Y et al. Neuro-Oncology, 2009.