THE ROLE OF METABOTROPIC GLUTAMATE RECEPTOR 1 (MGLU1) DEPENDENT SIGNALING IN GLIOMA CELL LINE VIABILITY

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THE ROLE OF METABOTROPIC GLUTAMATE RECEPTOR 1 (MGLU1) DEPENDENT SIGNALING IN GLIOMA CELL LINE VIABILITY

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ABSTRACT

Glioma refers to malignant central nervous system tumors that have histological characteristics in common with glial cells. The most prevalent type, glioblastoma multiforme, is associated with a poor prognosis and few treatment options.

Several lines of evidence suggest that derangements in the control of local glutamate concentration and signaling at various glutamatergic receptors promote glioma viability and growth. Considerable research has been conducted to study the role of glutamate transporters, as well as ionotropic and group II metabotropic receptors in glioma. However, only one previous study has focused on the specific role of the metabotropic glutamate 1 (mGlu1) receptor in glioma.

Based on reports of aberrant expression of mGlu1 receptor mRNA in glioma, evidence that melanoma growth is directly influenced by the mGlu1 receptor, and our laboratory’s characterization of β-arrestin dependent pro-survival signaling by this receptor, this dissertation investigates the hypothesis that glioma cell lines aberrantly express the mGlu1 receptor and depend on mGlu1 receptor mediated signaling to maintain viability and proliferation.

Three glioma cell lines (Hs683, A172 and U87) were tested to confirm that mGlu1 receptor mRNA was expressed in glioma cells (in contrast to astrocytes), and that glioma cells both secrete high concentrations of glutamate, and depend on the continuous presence of glutamate for viability. Pharmacologic and genetic evidence is presented that suggests mGlu1
receptor signaling specifically supports glioma proliferation and viability. For example, selective non-competitive antagonists of the mGlu1 receptor, CPCCOEt and JNJ16259685, decreased the viability of these cell lines in a dose dependent manner and GRM1 (mGlu1 gene) silencing significantly reduced glioma cell proliferation. Also, results of an anchorage-independent growth assay suggest that noncompetitive antagonism of the mGlu1 receptor may decrease the tumorigenic potential of Hs683 glioma cells. Finally, data are provided that support the hypothesis that a β-arrestin dependent signaling cascade may be involved in glutamate-stimulated viability in glioma cells and that ligand bias may exist at mGlu1 receptors expressed in these cells. Taken together, the results presented in this dissertation strongly suggest that the mGlu1 receptor may act as a proto-oncogene in glioma and be a viable drug target in glioma treatment.
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Akt (Protein kinase B)
ATCC (American Type Culture Collection)
AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)
ANOVA (Analysis of Variance)
cDNA (complementary deoxyribonucleic acid)
CHO (Chinese hamster ovary)
CGN (Cerebellar granule neurons)
DMEM (Dulbecco’s modified eagle medium)
DHPG (3,5-Dihydroxyphenylglycine)
DMSO (Dimethyl sulfoxide)
EAAT1/2 (Sodium and potassium-dependent excitatory amino acid transporters)
EGFR (Epidermal growth factor receptor)
ERK (Extracellular-signal regulated kinase)
FBS (Fetal bovine serum)
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)
GPCR (G protein coupled receptor)
GBM (Glioblastoma Multiforme)
GluA (AMPA receptor subunit)
GPT (Glutamate pyruvate transaminase)
GRM1 (Glutamate metabotropic receptor 1 gene)
HRP (Horseradish peroxidase)
RT- PCR (Reverse transcription polymerase chain reaction)
SAGE (Serial analysis of gene expression)
SEM (Standard error of the mean)
shRNA (Small/short hairpin ribonucleic acid)
siRNA (Small/short inhibitory ribonucleic acid)
SK2 (SK-MEL-2)
SK5 (SK-MEK-5)
System xc⁻ (cystine/glutamate antiporter)
TCGA (The Cancer Genome Atlas)
TMD (Transmembrane domain)
TMZ (Temozolomide)
VEGF (Vascular endothelial growth factor)
WHO (World Health Organization)
WT (Wild type)
CHAPTER 1
INTRODUCTION

1.1 Glioma is associated with a poor prognosis and limited treatment options.

The term glioma refers to malignant central nervous system tumors that have histological characteristics in common with glial cells. Astrocytomas account for 80% of malignant brain tumors, followed by oligodendroglioma, oligoastrocytoma, and ependymal tumors. The World Health Organization Grading System (WHO I-IV) is often used to characterize clinically diagnosed glioma. WHO grades are based on malignant characteristics such as the degree of atypical morphology, mitotic activity, vascularization, and necrotic area. Grades I (i.e. pilocytic astrocytoma) and II (i.e. diffuse astrocytoma, oligodendroglioma, ependymoma) are defined as low-grade tumors, whereas Grades III (i.e. anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic ependymoma) and IV (i.e. glioblastoma multiforme) are considered high-grade tumors. WHO grades are helpful in determining prognosis. For example, Grade I astrocytoma has a five-year survival rate of approximately 90%, while Grade IV astrocytoma has a five-year survival rate of less than 5%. Unfortunately, low-grade tumors often progress to WHO grade III and IV tumors over time. In addition to dramatically reducing life expectancy, gliomas significantly impair a patient’s quality of life. Common symptoms include nausea, vomiting, headaches, memory impairment, personality changes and seizures.

Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most common form of glioma, with approximately 10,000 patients diagnosed each year in the United States. The
current standard of care for GBM involves radiation therapy, aggressive surgical resection, and temozolomide (TMZ) treatment. However, even with the widespread use of these treatments, GBM survival rates are reported to be only 12.2-15.9 months following diagnosis.

Radiation was determined to have a dose-response survival benefit compared to no treatment over three decades ago. A trial comprising 621 patients demonstrated a median survival time of 18 weeks with no treatment, and a median survival time of 42 weeks with a dose of 6000 rad. Surgical resection has been shown to extend life expectancy by an additional four months. It is also useful in providing a biopsy for definitive diagnosis and symptomatic relief from tumor mass effects. Unfortunately, the benefits of surgical resection are limited by the diffuse pattern of these tumors. This pattern makes it impossible to remove all malignant cells without causing unacceptable damage to the surrounding brain. Therefore, despite aggressive resection, recurrence almost always occurs in WHO grade II-IV tumors. Also, repeat resections are not a viable long-term treatment option because they are associated with a high degree of morbidity and mortality.

The alkylating chemotherapeutic agent temozolomide became the standard of care for malignant glioma after a randomized, multicenter, phase 3 trial found a 2-year survival increase from 11.2%-27.3%, and a 5-year survival increase from 1.9%-9.8% when TMZ was administered to patients concurrently receiving radiotherapy. TMZ is most effective when there is promoter methylation of the DNA repair gene, MGMT (O6-methylguanine-DNA-methyltransferase) in the tumor cells. Unfortunately, only 44% of GBMs present with MGMT methylation, which diminishes the impact of this drug on overall survival.
These three treatments clearly provide some survival benefit. However, they do little to curb the devastating impact of this disease. GBMs continue to account for the highest number of years of life lost among malignant tumors. Therefore, there is an urgent need to identify pharmacological targets that could lead to more effective treatments.

1.2 While experimental glioma treatments demonstrate promise, the development of resistance consistently limits treatment effectiveness.

A common experimental approach to treating glioma has been to identify tumorigenic signaling pathways and pharmacologically inhibit pathway components. GBM was one of the first types of cancer to be characterized using the Cancer Genome Atlas (TCGA) approach. This approach involved the collection of over 200 independent GBM specimens and the performance of several large-scale analyses to identify common patterns of genetic and epigenetic abnormalities. Using the data from these studies, “gene expression subtypes” were identified including one characterized by loss or mutation of neurofibromin 1, another characterized by aberrant epidermal growth factor receptor (EGFR) expression or signaling, and a third with aberrant platelet-derived growth factor receptor alpha (PDGFR) and isocitrate dehydrogenase genes. Additional signaling pathways identified as potential therapeutic targets include the phosphatidylinositol-3-OH kinase (PI3K) pathway, vascular endothelial growth factor (VEGF), and the p53 and retinoblastoma (Rb) protein pathways.

The authors of the TCGA studies suggest that in-depth molecular characterization of individual GBM tumors and targeted treatment will offer superior patient outcomes in the future.
However, there is increasing evidence that successful treatment of glioma will require a multimodal approach that addresses the heterogeneous nature of glioblastoma and overcomes the redundancy built into signaling pathways that sustain tumor growth. Previous efforts to target epidermal growth factor receptor signaling illustrate this point. EGFR mediated signaling is amplified in 40-50% of GBM cases. While several EGFR inhibitors and anti-EGFR antibodies were successful in reducing glioma growth in preclinical studies, these results were not replicated in clinical trials. It was found that when EGFR was pharmacologically inhibited or treated with anti-EGFR antibodies, populations of glioma cells with genetic or epigenetic changes to phosphatase and tensin homolog (PTEN) were selected for and/or PDGFR signaling was induced. Each of these responses provided a means for compensatory down-stream pro-survival signaling, conferring treatment resistance. Similarly, vascular endothelial growth factor (VEGF) showed therapeutic promise as a target against human GBM in xenograft animal models. However, when bevacizumab, a monoclonal antibody that binds and inactivates VEGF, was tested in clinical trials, patients rapidly developed resistance and suffered from a more invasive tumor phenotype upon recurrence. It was later determined that suppression of VEGF signaling enhances Met receptor tyrosine kinase activity which is known to promote invasive tumor growth. Thus, it appears when a single component of a signaling pathway is inhibited in glioma, compensatory responses occur within that signaling axis to sustain tumor viability and/or inhibition confers a selective advantage to glioma cell populations capable of maintaining viability through alternative cell signaling pathways.

Immunotherapy has generated considerable interest as an alternative therapeutic strategy in the treatment of GBM. Preclinical studies using several immunotherapy approaches show
promise. These include the use of dendritic cells loaded with tumor lysate, the use of vaccines targeting tumor-specific epitopes, the generation of chimeric antigen receptor T cells, and checkpoint inhibitors \(^{20,21}\). While these approaches are relatively new and few have completed phase II clinical trials, an early example suggests resistance could also be problematic in glioma immunotherapy. A phase III clinical study testing the effectiveness of a vaccination against an EGFR\(_{vIII}\) peptide was recently terminated after it was determined that survival endpoints would not be met \(^{22}\). The mechanisms for treatment failure have not been conclusively determined. However, one study found that EGFR\(_{vIII}\) positive tumors treated with an anti-EGFR\(_{vIII}\) vaccine no longer expressed the epitope on recurrence, \(^{19}\) indicating that immune therapy may also present the limitation of treatment resistance or “immunologic escape.” Thus, like EGFR and VEGF inhibitors, it is possible that GBM tumors will also evade treatment with immune therapies against singular targets. These examples indicate that GBM treatment capable of extending life beyond 16 months may require concurrent therapies targeting diverse and relatively independent mechanisms of tumor proliferation, survival, and migration. Therefore, it is important to continue investigating alternative glioma treatments that could be incorporated into a multimodal treatment strategy.

1.3 High extracellular glutamate concentration provides a selective advantage to glioma in the brain.

Glutamate is considered the primary excitatory neurotransmitter in the central nervous system and an important mediator of proliferation, migration, and survival during neuronal
development. The typical glutamate concentration in the brain is 1-3 μM. However, following synaptic glutamate release, glutamate concentrations are estimated to reach 1mM at the synapse and up to 190μM in perisynaptic regions. Further contributing to extracellular glutamate concentration is the cystine/glutamate antiporter (system x\textsubscript{c}\textsuperscript{-}). System x\textsubscript{c}\textsuperscript{-} activity involves the secretion of glutamate in exchange for uptake of cystine, the rate-limiting step in glutathione production. Rapid reuptake of synaptic and system x\textsubscript{c}\textsuperscript{-} secreted glutamate is critical to prevent over-activation of ionotropic glutamate receptors, which can result in excessive intracellular calcium levels and neuronal death, or excitotoxicity. In the normal brain, astrocytic high-affinity sodium and potassium-dependent excitatory amino acid transporters (EAAT1/2) are primarily responsible for this rapid reuptake.

In glioma, the balance between these systems is disrupted; synaptic reuptake is inhibited secondary to downregulation of EAAT1/2 expression and system x\textsubscript{c}\textsuperscript{-} is overexpressed leading to increased glutamate secretion into the extracellular space. It is, therefore, not surprising that glioma cells in vitro and GBM tumors in vivo have been reported to release high concentrations of glutamate. For example, Ye and colleagues observed glutamate concentrations in cultured glioma cells increasing from undetectable to over 400μM in a twelve-hour period. Similarly, when microdialysis catheters were implanted adjacent to GBM tumor resection margins in surgical patients, extracellular glutamate measurements were as high as 350 μM.

The release of high concentrations of glutamate provides glioma with a distinct survival advantage. While, neuronal ionotropic receptors become chronically overstimulated in this environment, resulting in excitotoxicity and neuronal death, glioma cells thrive in high concentrations of glutamate. The death of neurons provides space for glioma tumor growth.
within the constrained cranial compartment. System $x_2^{-}$ activity also enhances glioma viability by supporting increased production of the intracellular antioxidant glutathione. High intracellular glutathione levels provide glioma cells with protection from oxidative stress including that induced by chemotherapy and radiation therapy. Furthermore, several lines of evidence suggest glutamate activates ionotropic and metabotropic glutamate receptors aberrantly expressed on glioma cells, promoting signaling cascades that enhance glioma proliferation, migration, and survival.

1.4 Signaling through ionotropic and metabotropic glutamate receptors promotes the growth and survival of several malignancies, including glioma.

Glutamate activates two distinct receptor types: ligand-gated ionotropic receptors and G-protein coupled metabotropic receptors. Ionotropic receptors are further sub-classified as N-methyl-D-aspartate (NMDA), $\alpha$-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. NMDA receptor-mediated signaling has been implicated in the promotion of several types of cancer including colon, breast, lung, and glioma. In glioma, NMDA receptors appear to exert their primary effect by enhancing the excitotoxic death of adjacent neurons, providing space for continued tumor growth, rather than directly driving proliferation. For example, NMDA antagonists, memantine and MK801, slowed the growth of glioma tumor xenografts in vivo but did not influence proliferation.

AMPA receptor-mediated signaling has also been implicated in the promotion of several types of cancer including pancreatic, lung, leukemia, lymphoma, breast, colon, and...
glioma\textsuperscript{26,42,43}. As with NMDA receptors, AMPA receptors expressed in glioma cells contribute to excitotoxic neuronal death\textsuperscript{26}. However, AMPA receptors also appear to mediate other aspects of glioma viability. Ishiuchi and colleagues found that calcium-permeable AMPA subunits GluA1 and GluA4 were highly expressed in glioblastoma surgical specimens, in comparison to GluA2, a subunit that excludes calcium conductance. Furthermore, when they induced GluA2 expression via adenovirus or applied AMPA antagonists, decreased migration, increased apoptosis, and reduced levels of mitotic activity were measured, indicating that AMPA mediated calcium conductance mediated these effects\textsuperscript{44}. In a further study, the researchers linked calcium permeant AMPA signaling to Akt activation, a pathway known to promote survival and stimulate malignant growth\textsuperscript{42}.

Metabotropic glutamate receptor signaling also appears to be an important contributor to the proliferation, migration, and survival of a variety of malignancies\textsuperscript{24,32,45}. Eight metabotropic glutamate receptors have been described and classified into three groups based on similarities in sequence homology, G-protein coupling, and pharmacology\textsuperscript{46}. Group I includes the mGlu1 and mGlu5 receptors, which are traditionally associated with a G\alpha_q-protein mediated signaling cascade that involves phospholipase C activation, phosphoinositide hydrolysis, the activation of protein kinase C and increases in intracellular calcium concentration\textsuperscript{46}. Group II includes the mGlu2 and mGlu3 receptors; while Group III includes the mGlu4, mGlu6, mGlu7 and mGlu8 receptors. Group II and III receptors are traditionally associated with G\alpha_{i/o} signaling which involves the inhibition of adenylate cyclase and its downstream targets\textsuperscript{32}.

Among the metabotropic receptors, the group II receptors, mGlu2 and mGlu3, have been most extensively studied in glioma. mGlu3 receptor expression levels in GBM tumors have been
negatively associated with survival in surgical patients \(^{47}\). Furthermore, several groups have found that the selective mGlu2/3 orthosteric antagonist, LY341495, reduces glioma cell proliferation \textit{in vivo} and \textit{in vitro} \(^{47-49}\). In one example, LY341495 blocked tumor growth in models where U87 GBM cells were either injected subcutaneously or into the caudate nucleus of nude mice \(^{48}\). These anti-tumor effects have been attributed to mGlu3-mediated amplification of pro-survival cell signaling pathways including PI3K/Akt \(^{47,48}\), and MAPK/ERK1/2 \(^{50}\). It also appears that mGlu3 receptors stimulated by glutamate may promote maintenance of glioma cells in an undifferentiated state \(^{49}\).

1.5 Several lines of evidence suggest glutamate stimulation of aberrantly expressed mGlu1 receptors results in enhanced proliferation and survival of malignant cells.

Group I metabotropic receptors have also been associated with several types of cancer \(^{51}\). However, compared with group II receptors, they have received little attention in glioma research. Interestingly, mGlu5 receptor mRNA and protein expression have been confirmed in several glioma cell lines \(^{52,53}\) and in normal astrocytes \(^{54}\). Therefore, it is difficult to determine if mGlu5 receptors expressed in glioma actively contribute to aberrant malignant signaling or if detection of mGlu5 in glioma reflects the underlying expression profile of its astrocyte progenitor cell. Further investigation of mGlu5 receptor signaling in glioma should be considered based on evidence that mGlu5 receptor expression and activity contribute to poor outcomes and increased migration and invasion of at least one malignancy, oral squamous cell carcinoma \(^{55}\).
The other group I receptor, mGlu1, has not been detected in normal brain astrocytes and, while it is expressed in immature oligodendrocytes, expression levels greatly decline as the cells mature. Despite the lack of mGlu1 receptor expression in mature glial cells, several independent studies suggest that the mGlu1 receptor is expressed in malignant glioma. For example, when 26 pediatric brain tumor specimens (six medulloblastoma, eight ependymoma, four glioblastoma and eight low grade astrocytoma) were evaluated by semi-quantitative RT-PCR, mGlu1 receptor mRNA was highly expressed across various brain cancer subtypes, including GBM. In addition, these researchers found that mGlu1 receptor expression was most pronounced in high grade tumors associated with poor prognoses. Another study examined the expression of mGlu1 receptor mRNA in various malignant cell lines and detected the transcript in all three glioma cell lines tested (U87, U343, and MOGGCCM). Additionally, using high-throughput Serial analysis of gene expression (SAGE) analysis, mGlu1 mRNA expression was observed in a number of GBM tumor specimens along with a GRM1 (mGlu1 coding gene) missense mutation in one specimen. This finding was consistent with other high-throughput genetic studies that detected frequent GRM1 missense mutations in a wide variety of cancerous specimens.

The observation that gliomas, including high-grade astrocytomas, express mGlu1 receptor RNA, while normal brain astrocytes and mature oligodendrocytes do not, is particularly interesting when one considers the mGlu1 receptor’s likely role in driving tumor growth in malignant melanoma. Like astrocytes, melanocytes do not normally express the mGlu1 receptor. However, expression of GRM1 has been reported to be present in 80% of melanoma cell lines and 65% of melanoma biopsies. Furthermore, expression of GRM1 may promote the
transformation of melanocytes into a malignant melanoma phenotype. For example, when a full length mGlu1 receptor cDNA was cloned into melanocytes and then allografted into a mouse model, the mice formed invasive, highly vascularized tumors. When the clones were then treated with mGlu1 receptor siRNA, the tumorigenic phenotype was significantly reversed, indicating that mGlu1 expression was critical for maintenance of the malignant transformation and that GRM1 was acting as a proto-oncogene in melanoma. Additional in vivo and in vitro studies conducted in our laboratory have validated the oncogenic role of mGlu1 receptor signaling in melanoma. For example, treatment of melanoma cells with mGlu1 receptor shRNA and mGlu1 selective antagonists revealed that: 1) receptor stimulation by glutamate was required for sustained cellular viability and proliferation in cell culture and 2) that receptor signaling stimulated tumor growth in a murine xenograft model.

The possibility that GRM1 may act as a proto-oncogene in other types of cancer is worth consideration. Several independent studies suggest aberrant expression of mGlu1 receptor mRNA exists in a variety of malignant cells in addition to melanoma and glioma including, prostate carcinoma, lung carcinoma, colon adenocarcinoma, T-cell leukemia, and breast carcinoma. mGlu1 expression appears to have functional consequences in triple-negative breast carcinoma that parallel those in melanoma. For example, researchers have reported that inhibition of mGlu1 signaling through gene silencing techniques and selective mGlu1 receptor antagonists reduced the malignant growth of triple-negative breast carcinoma and promoted apoptosis in both cell culture and a xenograft model. Only one previous study has been conducted to characterize the role of the group I receptor, mGlu1, in glioma viability. This study suggested that inhibition of mGlu1 receptor signaling decreased viability, increased
apoptosis, and decreased migration of U87 glioma cells. Despite some limitations in study design (see discussion), these results support the idea that aberrantly expressed mGlu1 receptors may have a functional role in malignant glioma that is similar to its oncogenic effect in melanoma.

1.6 The mGlu1 receptor may promote proliferation, migration, and survival in malignant cells by activating a \( G_{\alpha q} \) independent, \( \beta \)-arrestin dependent signaling cascade.

Our laboratory has reported a mechanism by which glutamate-stimulated mGlu1 receptors mediate cell survival that could account for the mGlu1 receptor’s observed effects in melanoma, breast cancer and glioma. This mechanism involves a \( G_{\alpha q} \) independent, \( \beta \)-arrestin dependent signaling cascade that induces sustained ERK phosphorylation and subsequent improved viability in serum deprived cell cultures. These effects have been confirmed in several cellular models including CHO cells stably expressing the mGlu1 receptor, cerebellar granule neurons, and in mGlu1 receptor positive melanoma cells lines, SK2 and SK5. These findings indicate that this pro-survival signaling pathway may be active in a variety of mGlu1 receptor expressing cell types. Therefore, it is important to understand the role of \( \beta \)-arrestin dependent mGlu1 receptor signaling in other mGlu1 receptor positive cancer cells, such as glioma. If confirmed, it may reveal the receptor to be a novel drug target against multiple types of malignant growth. This could provide an opportunity to diversify treatment strategies and reduce the development of treatment resistance in glioma patients.
Based on reports of aberrant expression of the mGlu1 receptor mRNA in glioma, accumulating evidence that melanoma growth is directly influenced by mGlu1 receptor expression and signaling, and our laboratory’s characterization of β-arrestin dependent pro-survival signaling by this receptor, we investigated the possibility that mGlu1 receptors influence the proliferation and survival of three commonly used glioma cell lines. The Hs683 cell line was selected as a model for a highly infiltrative oligodendroglioma, while the U87 and A172 served as models for high-grade astrocytoma or GBM.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials.

Lombardi Comprehensive Cancer Center Tissue Culture Shared Resource at Georgetown University provided the H4, Hs683, U87, A172, and U118 glioma cell lines. Dulbecco’s Modified Eagle Medium (DMEM), antibiotic-antimycotic, amphotericin B (Fungizone), fetal bovine serum (FBS), dialyzed FBS, Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit, and MTT (thiazolyl blue tetrazolium) were purchased from Life Technologies (Carlsbad, CA). CyQUANT® cell proliferation assay kit and Lipofectamine™ 3000 Reagent was purchased from ThermoFisher Scientific (Grand Island, NY). Glutamate pyruvate transaminase (GPT) was purchased from Roche (Indianapolis, IN). Calcein AM was purchased from Sigma-Aldrich (St. Louis, MO). Glutamate, aspartate, quisqualate, CPCCOEt, JNJ16259685, dynasore, and MPEP were purchased from Tocris Bioscience (Bristol, United Kingdom).

2.2 Cell culture.

All glioma cell lines were cultured in DMEM media supplemented with 100X antibiotic-antimycotic (1ml/50 ml media), amphotericin B (2.5µg/ml), and either 10% fetal bovine serum (full media) or 10% dialyzed fetal bovine serum (dialyzed media). Dialyzed fetal bovine serum was treated by the manufacturer to remove all small molecules (MW <10,000), including
glutamate. Full media was prepared using DMEM that contained 4mM L-glutamine, while dialyzed media was prepared with L-glutamine free DMEM. The cells were grown at 37°C and 6% CO2. CHO cells were cultured under the same conditions with proline supplementation (4.5g/500ml) of the media.

2.3 Viability assays (MTT and Calcein AM).

Cells were plated in 100 μL of full media to achieve 50% confluency on a 96 well plate (7,000 cells per well for U87 cells, 9,000 cells per well for Hs683 and A172 cells). The following day, the media was changed to 100 μL of full media or dialyzed media containing treatments as indicated (i.e. glutamate, quisqualate, CPCCOEt, JNJ16259685, MPEP, GPT, dynasore). A DMSO concentration of 1% was maintained in all treatment and control wells. The cells were incubated under treatment conditions for an additional 5 days. 1) MTT (thiazolyl blue tetrazolium) (Life Technologies, Carlsbad, CA) was then used to assess cell viability. Following the five-day incubation period, cells were incubated for 40 minutes in 0.2mg/ml of MTT. The MTT was then removed and 70 μl of DMSO was added to solubilize the cells and release the formazan product. The product was then read on the Envision plate reader (Perkin-Elmer, Waltham, MA). 2) Calcien AM is a lipophilic compound that produces a green fluorescence when hydrolyzed by intracellular esterases in intact (live) cells. Cells were counted, plated, treated and incubated as above. Five days after treatment application, the media was removed and 200 μl of 1 μM calcein AM was added to the cells in warm PBS. After a 30-minute incubation period at 37 °C, each well’s fluorescence was quantified on the plate reader with an
excitation wavelength of 485 nm and an emission wavelength of 530nm.

2.4 Measurement of glutamate concentration.

Measurement of glutamate concentration was accomplished using the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit (Life Technologies) without the amplification step. This assay is based on the following reaction: L-glutamic acid in the presence of glutamate oxidase generates alpha-ketoglutarate, ammonia, and hydrogen peroxide. Hydrogen peroxide and Amplex red reagent react in the presence of HRP to produce the fluorescent product, resorufin. U87 (7000 cells/well), A172, and Hs683 (each 9,000 cells/well) cells were plated on 96 well plates in 100 µl per well of full media. Twenty-four hours later, the cells were washed with PBS twice and 100 µl per well of dialyzed media lacking glutamate was applied. The media was collected on days 1, 3, and 5 and stored at -20°C. After all samples had been collected, glutamate concentration was determined by adding 50 µl of reagent mixture (100µM Amplex® Red, 0.25U/mL horse radish peroxidase (HRP), and 0.08 U/mL of L-glutamate oxidase in 10mM Tris-HCl, pH 7.5) to 50 µl of (5-fold) diluted sample media. After an hour of incubation, fluorescence was quantified on the plate reader with an excitation wavelength of 570 nm and an emission wavelength of 585 nm. The values obtained were then fit to a standard glutamate concentration curve (Figure 1).
2.5 Glutamate Pyruvate Transaminase (GPT).

Unless otherwise indicated, GPT was applied to cell cultures in 100 µl per well of fresh media at a concentration of 35 µg/ml with 10 mM pyruvate, 24 hours after plating. GPT reduces the glutamate concentration by catalyzing the metabolism of glutamate and pyruvate to alpha-ketoglutarate and alanine. After a five-day incubation period, an MTT assay was used (as above) to quantify the GPT effect.

2.6 Detection of mGlu1 mRNA.

Total RNA was extracted from glioma cells cultured on 60mm dishes using the NucleoSpin RNA Plus protocol (Machery-Nagel, Bethlehem, PA) according to the manufacturer’s instructions. Extracted RNA was measured using a nanophotometer (IMPLEN, Munich, Germany). Equal concentrations of RNA were reverse transcribed to cDNA using Life Technologies High Capacity cDNA Reverse Transcription Kit and protocol. Next, reverse transcription polymerase chain reaction (RT-PCR) was performed to detect mGlu1 receptor expression in the various samples using Phusion High-Fidelity DNA Polymerase Kit and protocol (New England Biolabs, Ipswich, MA). mGlu1 receptor primers were designed to detect mGlu1a (amplicon 650 base pairs) and mGlu1b (amplicon 735 base pairs) as described previously. Briefly, the mGlu1 primer sequences used spanned exons VIII to X of the glutamate metabotropic 1 protein coding gene (GRM1). The difference in amplicon size between the splice variants is expected because mGlu1b contains an 85-base pair exon (IXC) between
exons VIII and X that is absent in mGlu1a mRNA. The primer sequences used were forward 2661: ATGCCCATTCTGTCTACCCCAAGT and reverse 3286 GCTCTGGCAAGAGCCTGACCTTTTC. Pilot experiments indicated signal saturation between 37 and 38 cycles. Therefore, after initial denaturation for 30 seconds at 98 °C, 36 cycles were performed: 98 °C for 10 seconds followed by 72 °C for 1 minute. After the 36th cycle, the samples underwent a final extension at 72 °C for 10 minutes. GAPDH expression was measured in a separate reaction using an equivalent volume from the same aliquot of cDNA used in the mGlu1 PCR reaction. The GAPDH forward primer sequence was: CAACCCAGAAGACTGTGGAT and the reverse primer sequence was: TTCTAGACGGCAGGTTCAGGT (amplicon 203 base pairs). Pilot experiments indicated signal saturation between 21 and 22 cycles. Therefore, after initial denaturation for 30 seconds at 98 °C, 20 cycles were performed: 98 °C for 10 seconds followed by 68°C for 30 seconds, followed by 72 °C for 30 seconds, with a final extension after the 20th cycle at 72 °C for 10 minutes. Reaction products were then loaded and run on 2% agarose gels. A CHO cell line stably transfected with human mGlu1 was used as a positive control.

2.7 mGlu1 receptor shRNA transfections.

Five psi-U6 plasmids containing the reporter gene eGFP and a puromycin resistance gene were purchased from GeneCopoeia (Rockville, MD). One plasmid served as a scrambled shRNA control while the other four contained shRNA sequences targeting GRM1 as follows: (starting base listed) 722 (cacgttggataagatcaac), 1507 (aggtcaggtcatttgatga), 1978 (gagtgctgaacattgatga),
and 2690 (ggaagtctaccttatctgc). Glioma cells were plated to achieve approximately 80% confluency on 60mm dishes and incubated overnight at 37°C and 6% CO₂. The following day, the cells were transfected with either a combination of four plasmids targeting the mGlu1 receptor mRNA (total 5µg cDNA), the plasmid containing scrambled shRNA (5µg cDNA), or treated with transfection agent only (vehicle). Lipofectamine™ 3000 Reagent was used following the manufacturer’s suggested transfection protocol, omitting the P3000 Reagent step.

2.8 Proliferation assay (CyQUANT® Cell Proliferation Kit).

One day after transfection with mGlu1 shRNA plasmids, control scrambled shRNA plasmid, or vehicle, the cells were plated on 96-well plates at 6,500 cells/well and incubated at 37°C and 6% CO₂. At 24, 48, and 72 hours, plates were withdrawn from the incubator, the media was removed, and the plates were stored at -80°C. After all samples were collected and stored for a minimum of 24 hours at -80°C, they were thawed to room temperature and 200 µl of a 1X mixture of Cyquant®GR dye in Cyquant® cell lysis buffer diluted in distilled water was applied to the cells. Cyquant GR contains a proprietary cyanine dye that emits a fluorescent signal when bound to cellular nucleic acids. Measurement of emitted fluorescence allows assessment of DNA content over time, which will reflect proliferative activity. After a 5-minute incubation period, each well’s fluorescence was quantified on a microplate reader with an excitation wavelength of 485 nm and emission wavelength of 520 nm. A standard curve using a serial dilution of Hs683 glioma cells confirmed cell densities were in the linear range of detection (Figure 2).
2.9 Anchorage-Independent growth.

Anchorage-independent growth was measured using the Soft Agar Colony Formation Assay described by Borowicz and colleagues. In brief, 1.5 ml of 0.5% noble agar in DMEM media containing either 1% DMSO, 30µM, 100µM, or 300µM JNJ16259685 was plated onto a 6-well plate to form a bottom layer. After allowing 30 minutes at room temperature for bottom layer solidification, 10,000 Hs683 cells per well were plated in 1.5 ml of 0.3% noble agar in DMEM media in either 1% DMSO, 30µM, 100µM, or 300µM JNJ16259685 over the bottom layer of agar with matching treatment conditions. Following an additional 30 minutes at room temperature, the plates were moved to an incubator set at 37°C and 6% CO2. Twice per week, 100 µl of DMEM and the respective treatment (DMSO only, 30, 100, or 300 µM JNJ16259685) were added to the wells to replace evaporative losses. After 21 days, 200µL of nitroblue tetrazolium chloride was applied to each well to stain the colonies present and the plates were placed back in the incubator overnight. The following day, the plates were photographed and analyzed using Image J, which generated values for colony number and colony area based on detection of blue color. Average colony size was calculated using (sum of colony area per well)/(number of colonies per well). A grand mean for each experiment was calculated and used to normalize the data within each individual experiment. A total of 3 (100µM) or 4 independent experiments (DMSO, 30, 300µM JNJ) were conducted, each measured in duplicate. For the final presentation, the means from the independent experiments were averaged and normalized to the mean of the control group (DMSO).
2.10 Construction of “forced” monomer mGlu1 receptors (future directions).

These constructs were developed using the method reported to disrupt GABA B receptor dimerization. The receptors were mutated to express N-glycosylation sites at the LBD dimer interface that serve to physically occlude dimerization through addition of a “glycan-wedge.” Sequence homology between the GABA B and mGlu1 receptor LBDs was utilized to compare the dimer interface sequences. mGlu1 receptor residues corresponding to GABA B forced monomer mutation sites were successfully identified. Next, molecular modeling was used to visualize the orientation of each candidate residue and three promising residues were identified: S166, Q170, and L177. PCR primers were designed to introduce a series of point mutations equivalent to the three residue N-glycosylation consensus sequence NXS where X is any residue except proline. Additionally, a QXS control mutation, which would not result in N-glycosylation, was also designed to account for any disruptions made by the mutation alone. These constructs (Table 1) were introduced into a N-terminal FLAG epitope-tagged mGlu1 receptor, cloned into a pIRES/ACGFP1 vector, and confirmed by sequencing analysis. CHO cells were then transfected and successful clones verified and stabilized based on GFP expression.

2.11 PI hydrolysis (future directions).

Untransfected (UT) and stably transfected (forced monomer: N166, N170, N177, control mutant: Q166, Q170, Q177, and WT mGlu1) CHO cells were cultured in 96-well plates at 50,000 cells/cm² cells were grown to 90% confluency and then incubated overnight with 0.625
μCi/well [³H] inositol. After washing, receptor ligands (3 mM glutamate and/or 300 μM of the noncompetitive mGlu1 receptor antagonist JNJ16259685) were incubated for 1 hour at 37 °C in Locke’s buffer with 20mM LiCl. The media was then aspirated and 10mM formic acid was used to extract the inositol phosphates. The samples were then incubated with polylysine coated yttrium scintillation proximity assay beads, and then scintillation counting was used to detect inositol phosphates after a 600-minute delay.

2.12 Statistical analysis.

Prism 7.0 (GraphPad Software, San Diego, CA) was used to model non-linear regression using the variable slope, four-parameter equation. One way ANOVA using Dunnett’s Multiple Comparisons Test or Tukey’s Multiple Comparisons Test was used when comparing multiple groups. Statistical significance was set at *p<0.01.
Figure 1. Representative standard curve generated by Amplex® Red Glutamic Acid/Glutamate Oxidase Assay in the presence of serial dilutions of glutamate (range: 1.15-66.66 \( \mu \text{M glutamate} \)). The data were fit to a line with the following equation: \( y=2.0882x + 7.7667 \) \((r^2 = 0.986)\) using Microsoft Excel (Redmond, WA).

Figure 2. A standard curve using a serial dilution of Hs683 glioma cells confirmed cell densities were in the linear range of detection in the CyQUANT cell proliferation assay. As seen in Figure 16, RLU values utilized in determining relative concentration of DNA between samples were within the linear range of detection (RLUs <16).
CHAPTER 3

RESULTS

3.1 The continuous presence of glutamate promotes glioma cell line viability.

Our first series of experiments were designed to test the dependence of glioma cell line viability on the continuous presence of glutamate in the culture media. Glioma cells were incubated in standard media conditions (full media containing glutamate), media lacking glutamate (dialyzed media), and dialyzed media supplemented with increasing concentrations of glutamate (Figure 3). Following a five-day incubation period, cellular viability was measured using the MTT assay. As shown in Figure 3, Hs683 glioma cell line viability dose dependently increases in the presence of glutamate ($EC_{50}$ 3.7 mM (95% CI: 2.3-6.0)). This value was consistent with the $EC_{50}$ of glutamate reported to promote viability in melanoma cell lines known to aberrantly express the mGlu1 receptor, SK2 ($EC_{50}$ = 4.3 mM) and SK5 ($EC_{50}$ 3.4 mM) \(^\text{66}\). Furthermore, the cytoprotective effect of glutamate was observed in all glioma cell lines tested. When incubated in dialyzed media, Hs683, A172, and U87 cell line viability was reduced to 37% ($\pm$ 4), 45% ($\pm$ 7), and 48% ($\pm$ 11) of full media controls, respectively (Figure 6). However, when dialyzed media was supplemented with 20mM glutamate, viability returned to 79% ($\pm$ 13), 75% ($\pm$ 9), and 90% ($\pm$ 7), of full serum controls in Hs683, A172, and U87 cell lines, respectively (Figure 6).

Recent reports indicate that the MTT assay may have off-target effects leading to inaccurate cellular viability measurements under some treatment conditions \(^\text{80,81}\). For example,
ethanol induced oxidative stress has been shown to enhance intracellular NADH concentrations which can then reduce tetrazolium salts and produce an exaggerated result. Therefore, we confirmed our results in two alternative cellular viability assays that are not directly influenced by mitochondrial function; the Calcein AM assay which measures esterase activity in intact (live) cells and the CyQUANT® Cell Proliferation assay which measures nucleic acid content (an indirect measure of cell number). When A172 cells were incubated for five days under treatment conditions, Calcein AM and MTT assays provided similar results when measuring the effect of glutamate supplementation on cellular viability (Figure 7A and C). Likewise, when Hs683 viability was measured using the CyQUANT® assay (after three days of treatment incubation), the results were similar to viability measured by MTT (after five days of treatment incubation) (Figure 7B and D). Therefore, it appeared MTT was providing an accurate assessment of glioma cell viability in this model.

The release of glutamate by glioma cells is well documented in the literature. We confirmed this finding and quantified the release of glutamate by cell lines used in this study (Figure 4). Glutamate concentrations measured 5 days after plating in dialyzed media were 278 \( \mu M \) (\( \pm \) 22), 235 \( \mu M \) (\( \pm \) 27), and 246 \( \mu M \) (\( \pm \) 23), in Hs683, A172, and U87 cells, respectively. Concentrations within this range have been shown to promote survival in cells confirmed to express high levels of the mGlu1 receptor, such as CHO cells heterologously expressing the mGlu1 receptor (EC\(_{50}\) = 153 \( \mu M \)) and cerebellar granule neurons (EC\(_{50}\) = 76\( \mu M \)) \(^{68,70} \). Therefore, it is likely that glutamate released over a five-day incubation period could partially rescue glioma cells cultured in dialyzed media, decreasing the sensitivity of the assay.

To address this confounding factor, glutamate pyruvate transferase (GPT) was added to
full media to enzymatically remove glutamate as it was released (Figures 5 and 6). GPT removes glutamate by catalyzing the metabolism of glutamate and pyruvate to alpha-ketoglutarate and alanine. As expected, when GPT was added to the media, Hs683 viability was dose dependently reduced (IC$_{50}$ 28 µg/ml (CI 95% (4-181)) (Figure 5). The dependence on glutamate, as revealed by GPT, was observed in all cell lines tested. Viability was reduced to 27%($\pm$ 5), 41%($\pm$ 4), and 34%($\pm$ 4) of full serum controls in Hs683, A172, and U87 cell lines, respectively (Figure 6). Not surprisingly, viability was lower in GPT treated cells compared with cells cultured in dialyzed media only (Figure 5 and 6). Presumably, this enhanced viability in dialyzed media reflects an autocrine effect of the glutamate released from the glioma cells over the five-day incubation period. This effect was prevented in GPT containing wells where the released glutamate was enzymatically cleared from the media upon release. Taken together, these results indicate that these glioma cell lines are dependent on glutamate for sustained viability; this adds validity to the argument that pharmacological antagonism of glutamatergic signaling should be explored as a potential treatment for glioma.

3.2 mRNA encoding the mGlu1 receptor was confirmed in five human glioma cell lines by reverse transcription polymerase chain reaction (RT-PCR).

Using RT-PCR, we tested for the expression of mGlu1 mRNA in multiple glioma cell lines. Primers designed to detect mGlu1a and mGlu1b mRNA transcripts produced two distinct bands at the appropriate base pair weights in H4 (low-grade glioma), Hs683 (oligodendroglioma), U87 (GBM/astrocytoma), A172 (GBM/astrocytoma), and U118
(GBM/astrocytoma) cell lines (Figure 8). The expression level varied among the cell lines. For example, H4 cells produced an mGlu1a band just above the threshold of detection, while robust mGlu1a signals were observed in the other cell lines (Figure 8). However, the band intensity of the loading control, GAPDH, was consistent, indicating equal starting concentrations of cDNA. These results suggest that the mGlu1 gene, GRM1, is actively transcribed in human derived glioma cells and that the receptor transcription level varies among the various cell lines. It would have been ideal to also confirm the expression of the mGlu1 receptor protein. Unfortunately, because we found that all currently available commercial mGlu1 receptor antibodies lacked specificity for the human mGlu1 receptor, we were not able to successfully complete these experiments. Should an effective human mGlu1 receptor antibody become available, it would be important to confirm protein expression.

3.3 β-arrestin dependent mGlu1 receptor signaling may contribute to glioma cell line viability.

Studies conducted in our laboratory have suggested a positive impact of β-arrestin dependent mGlu1 receptor signaling on cellular viability in several models including CHO cells heterologously expressing the mGlu1 receptor, cerebellar granule neurons, and in melanoma. In addition, these studies have consistently concluded that ligand bias exists at the mGlu1 receptor and that: 1) unbiased ligands, such as glutamate and aspartate, can activate either the canonical Gαq pathway or the β-arrestin-dependent pathway and 2) biased ligands such as quisqualate and DHPG can only activate the Gαq pathway. Therefore, we tested if mGlu1
receptor signaling in glioma followed a similar pattern. Hs683 cell lines were cultured in full media (viability= 100%), dialyzed media (viability = 36% (± 2)), or dialyzed media in the presence of various mGlu1 receptor agonists for five days and then subjected to the MTT assay (Figure 9). As expected, 20 mM glutamate acted as a full agonist in dialyzed media with viability measured at 84% (± 5) of full serum controls. Aspartate acted as a less potent or efficacious agonist in dialyzed media with a viability 67% (± 3) of full serum controls. However, Gαq biased agonists, DHPG and quisqualate, did not significantly promote Hs683 cell line viability even when used in concentrations 100 and 300 fold higher than their reported EC₅₀, respectively (DHPG EC₅₀ = 10µM and quisqualate EC₅₀ = 1µM) ⁶⁹. For example, 1mM DHPG treated cells had a viability of 31% (± 2) and 300µM quisqualate treated cells had a viability of 38% (± 6) compared to full serum controls. When this experiment was repeated in A172 and U87 cell lines, the results were consistent (Figure 9). Below, we present pharmacologic and genetic evidence indicating that the mGlu1 receptor specifically mediates glioma cell viability. In this context, these agonist viability results suggest that ligand bias may also occur at mGlu1 receptors expressed on glioma cells.

To further investigate the possibility that β-arrestin dependent signaling was contributing to the effect of glutamate on glioma cell viability, the effects of dynasore on viability were measured. Dynasore inhibits the GTPase activity of dynamin, a critical player in clathrin mediated endocytosis, which is a prerequisite step in β-arrestin dependent signaling ⁸³. We found that dynasore dose dependently blocked the positive effect of glutamate on glioma cell line viability in Hs683 cells with an IC₅₀ of 31.8µM (CI 95% (24.1-42.1)) (Figure 10). In contrast, when increasing concentrations of dynasore were added to dialyzed media (with no supplemental
glutamate and GPT added to enzymatically remove secreted glutamate), the data could not be fit to a curve. This finding suggests dynasore’s effect was associated with blockade of a glutamate mediated effect and not a non-specific toxicity.

3.4 In the presence of glutamate, selective antagonists of the mGlu1 receptor negatively impact the viability of glioma cell lines in a dose dependent manner.

After confirming active transcription of the mGlu1 receptor gene in multiple glioma cell lines and the dependence of glioma cell line viability on non-biased mGlu1 agonists such as glutamate and aspartate, we then examined the effect of mGlu1 receptor antagonists in this model. Hs683 cells cultured in full media and treated with increasing concentrations of the non-competitive mGlu1 antagonist JNJ16259685 (JNJ), demonstrated reduced viability with an IC$_{50}$ of 192 µM (CI 95% (138-266)) (Figure 11A). Similarly, when Hs683 cells were cultured in dialyzed media supplemented with 20 mM glutamate, increasing concentrations of JNJ reduced viability with an IC$_{50}$ of 182 µM (CI 95% (152-218)) (Figure 11B). These IC$_{50}$ values were similar to the reported IC$_{50}$ for inhibition of viability in mGlu1 receptor positive melanoma cell lines SK2 (109µM) and SK5 (105µM) and in cerebellar granule neurons (19.8µM) 70. A representative photograph of Hs683 cells subjected to the various treatment conditions is presented in Figure 12. A second non-competitive antagonist, CPCCOEt, also inhibited Hs683 viability when added to dialyzed media containing 20 mM glutamate with an IC$_{50}$ of 321 µM (CI 95% (271-381)) (Figure 11C). Conversely, when increasing concentrations of JNJ (Figure 11B) or CPCCOEt (Figure 11C) were applied to the cells in dialyzed media without supplemental
glutamate and with GPT added to enzymatically remove all secreted glutamate, the data did not fit to a dose response curve. This suggests the antagonists’ reduction in viability was not the result of a non-specific toxicity and could be attributed to antagonism of mGlu1 receptor signaling.

Our findings were consistent in all glioma cell lines tested. JNJ and CPCCOEt were tested in two concentrations in the presence of 20 mM glutamate and dialyzed media (Figure 13). In A172 cells, 100 µM and 300 µM JNJ significantly reduced the protective effect of glutamate with viabilities measured at 58% (± 7) and 21% (± 3) respectively, compared with full media controls. Similarly, CPCCOEt at 100 µM and 350 µM significantly reduced the protective effect of glutamate with viability measured at 46% (± 14) and 22% (± 5), compared with full serum controls. In U87 cells, 100 µM and 300 µM JNJ reduced the protective effect of glutamate with viabilities measured at 80% (± 6) and 17% (± 2) compared with full serum controls. Similarly, CPCCOEt at 100 µM and 350 µM reduced the protective effect of glutamate with viability to 66% (± 9) and 32% (± 2) compared with full serum controls.

While the mGlu1 receptor has not been shown to be expressed in astrocytes or to be functionally relevant to their physiology, the other group I metabotropic receptor, mGlu5, which shares considerable sequence homology with the mGlu1 receptor, is highly expressed in astrocytes. The mGlu5 receptor has also been observed to be up-regulated in astrocytes in the context of pathological conditions including epilepsy, multiple sclerosis, and amyotrophic lateral sclerosis. Therefore, it was important to determine if mGlu5 receptor signaling was contributing to glutamate’s observed survival benefit in glioma cells. To test this possibility, MPEP, a selective, non-competitive, mGlu5 receptor antagonist, was applied to Hs683, A172,
and U87 cells in concentrations 10 and 1000-fold higher than MPEP’s reported IC\textsubscript{50} at mGlu5 (36nM)\textsuperscript{84}. As illustrated in Figure 14, 300 nM and 30µM MPEP did not significantly block the protective effect of 20 mM glutamate in any cell line tested. Therefore, the group I antagonist data indicate that glutamate’s protective effect may be selectively modulated by the mGlu1 receptor and that it may be a specific target useful in decreasing glioma cell survival.

**3.5 mGlu1 receptor shRNA reduces glioma cell line proliferation.**

Having established that selective mGlu1 receptor antagonists decrease glioma viability, we then utilized a gene silencing approach to confirm our pharmacological data. Four plasmids, each encoding shRNA targeting mGlu1 in a different location, were transfected into Hs683 glioma cells. A plasmid containing a scrambled shRNA was used to assess non-specific plasmid toxicity. Transfection of the plasmid was confirmed by GFP signal and gene knock-down was evaluated using RT-PCR (Figure 15). As expected, mGlu1a and mGlu1b amplicons were detected in Hs683 glioma cells transfected with a scrambled shRNA control plasmid. In contrast, mGlu1a and mGlu1b were effectively silenced in Hs683 glioma cells transfected with 4 different mGlu1 targeted shRNA plasmids.

Following confirmation of effective mGlu1 receptor knock-down, an attempt to establish a stable cell line was made using increasing concentrations of puromycin, selecting for transfected cells containing the plasmids’ puromycin resistance gene. However, despite a robust initial GFP signal, the cells did not survive the selection process, consistent with the hypothesis that the mGlu1 receptor is important for glioma cell viability. Therefore, we shifted to a transient
transfection approach. The five-day incubation period required to optimally detect viability differences using the MTT protocol was not appropriate considering the short duration of transient transfection. Therefore, the CyQUANT® Cell Proliferation assay, which has adequate sensitivity to detect differences three days after shRNA transfection was used (Figure 7B).

Using this assay, we measured proliferative activity over a 3-day period in 96 well plates and found that Hs683 cells treated with mGlu1 shRNA had significantly less nucleic acid content per well compared with those treated with a scrambled shRNA control or with transfection agent alone (Figure 16). No difference was noted between Hs683 cells exposed to transfection agent only and those transfected with the scrambled shRNA control plasmid (p=0.20), validating the specificity of the mGlu1 receptor shRNA effect. These results demonstrate that mGlu1 receptor knock-down specifically reduced the proliferation of glioma cells over time and strongly support the hypothesis that the mGlu1 receptor plays a significant role in the proliferative activity and viability of glioma cells \textit{in vitro}.

3.6 The mGlu1 antagonist, JNJ, dose dependently reduced anchorage-independent growth in Hs683 cells.

The ability of cells from cancerous tissues to form colonies in semi-solid media is a well-established predictor of tumorigenic and metastatic phenotypes in animals. Therefore, we tested the ability of Hs683 cells to grow in a soft agar colony formation assay. “Extreme” anchorage-independent growth has been defined as more than 500 colonies in a 6 well dish following a three week incubation in soft agar; while “extreme” anchorage-dependent growth has
been defined as less than 20 colonies \(86\). When we cultured Hs683 cells under these conditions, the cells exhibited significant anchorage-independent growth with an average colony number of 403 (± 89) per well and average colony size of 29µm\(^2\) (± 5) (Figure 17A and B). However, when the non-competitive mGlu1 antagonist JNJ was added to the semisolid media, it inhibited the ability of Hs683 to form colonies and decreased colony size in a dose dependent manner. When compared to control wells, a trend towards decreased average colony size (20µm\(^2\) (± 2)) could be seen with 30µM JNJ (p=0.10) before a decrease in colony number was observed. A significant difference in both colony number and size was seen when wells treated with 100 µM JNJ were compared to control, with an average colony number of 63 (± 13) per well and colony size of 12µm\(^2\) (± 2) (Figure 17 A and B). Furthermore, an average of 4 (± 1) colonies were detected when Hs683 was cultured with 300µM JNJ indicating a conversion to an “extreme” anchorage-dependent growth phenotype. These results suggest non-competitive antagonism of the mGlu1 receptor reduces the tumorigenic and metastatic potential of these glioma cells and predicts that it might be useful to decrease the growth of glioma in an in vivo model.
Figure 3. Hs683 glioma cell line viability dose dependently increases in the presence of glutamate. (EC50 3.7 mM (95% CI: 2.3-6.0)). The dashed line represents Hs683 cellular viability in dialyzed, glutamate free media. Cells were plated on day 1 in full media, treatment conditions were applied on day 2, and viability was measured utilizing the MTT assay on day 6. All data were normalized to viability in full media. Each data point represents the mean of 4 independent experiments (n) (± SEM) measured in triplicate. The data were analyzed with GraphPad Prism 7.0 using the non-linear regression, variable slope, four parameter equation.
Figure 4. Glioma cell lines produce significant concentrations of glutamate. Amplex® Red Glutamic Acid/Glutamate Oxidase Assay was used to measure glutamate concentration. Over a five-day period, glutamate concentration increased by 1.9, 2.2, and 3.8-fold in the culture media of Hs683, A172, and U87 cells, respectively. Each data point represents the mean of at least three independent experiments (n) (± SEM) measured in triplicate.
Figure 5. Glioma cell line viability is dose dependently reduced when glutamate-pyruvate transaminase (GPT) is added to the media in the presence of 10 mM pyruvate. (IC50 28ug/ml (CI 95% (4-181)). GPT reduces the glutamate concentration by catalyzing the metabolism of glutamate and pyruvate to alpha-ketoglutarate and alanine. The dashed line represents viability in dialyzed, glutamate free media. Cells were plated on day 1, GPT and pyruvate were added on day 2, and viability was measured utilizing the MTT assay on day 6. All data were normalized to viability in full media without GPT. Each data point represents the mean of at least three independent experiments (n) (± SEM) measured in triplicate. The data were analyzed with GraphPad Prism 7.0 using the non-linear regression, variable slope, four parameter equation.
Figure 6. The presence of glutamate promoted cell viability in all glioma cell lines tested. A significant difference in viability was found when glioma cells were cultured in full media compared with dialyzed media or full media with GPT. No, significant difference in viability was found between glioma cells cultured in full media compared with dialyzed media supplemented with 20mM glutamate. Each data point represents the mean of at least three independent experiments (n) (± SEM) measured in triplicate. One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01).
Figure 7. Calcein AM, CyQUANT, and MTT assays provided similar viability results. A significant difference in viability was found when glioma cells were cultured in full media compared with dialyzed media. However, no significant difference in viability was found between glioma cells cultured in full media compared with dialyzed media supplemented with 20mM glutamate. These findings were consistent in A172 cells measured using Calcein AM or MTT viability assays (A and C) and in Hs683 cells using CyQUANT and MTT viability assays (B and D). All data were normalized to cellular viability in full media. Bar graphs represent the mean of one or more representative experiments (± SEM). One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01, comparing to viability or proliferation in full media).
**Figure 8. Human glioma cell lines express mGlu1a & mGlu1b receptor mRNA.** (A) Representative 2% agarose gel containing RT-PCR products. As expected, mGlu1a was detected in CHO cells transfected with mGlu1a but human GAPDH was not. Both mGlu1a and mGlu1b were detected in all glioma cell lines. (B) PCR product intensity of this representative experiment was quantified using Image J and normalized to a matched human GAPDH control. GAPDH and mGlu1 are products of separate reactions that included equivalent volumes of the same aliquot of cDNA. Photographs were cropped to promote clarity.
Figure 9. Only unbiased mGlu1 receptor agonists promote glioma cell line viability. In glioma cells, glutamate acts as a full agonist promoting viability, aspartate acts as a less potent or efficacious agonist in Hs683 and U87 cells (A172 cells p= 0.2), while Gαq biased agonists DHPG and quisqualate do not promote glioma cell line viability. Bar graphs represent cell viability measured by the MTT assay and normalized to viability in full serum. Each condition, except full media, was in dialyzed serum. Each data point represents the mean (± SEM) of at least 3 independent experiments (n) measured in triplicate or more. One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01, comparing to viability in dialyzed media).
Figure 10. Blockade of receptor internalization with dynasore reduces glioma viability in a dose dependent manner. Dynasore dose dependently blocks glutamate’s positive effect of glioma cell line viability in Hs683 cells with an IC50 of 31.8μM (CI 95% (24.1-42.1)). In contrast, when increasing concentrations of dynasore were added to dialyzed media (with no supplemental glutamate and GPT added to enzymatically remove glioma secreted glutamate), non-linear regression did not fit the data. Each data point represents the mean (± SEM) of at least 3 independent experiments (n) measured in triplicate or more. Non-linear regression was used to fit the data using a variable slope, four parameter equation.
Figure 11. Selective non-competitive antagonists of the mGlu1 receptor decrease the viability of Hs683 cells in a dose dependent manner. (A) Hs683 cells cultured in full media and treated with increasing concentrations of the non-competitive mGlu1 antagonist JNJ16259685 (JNJ), demonstrated reduced viability with an IC\textsubscript{50} of 192 µM (CI 95% (138-266)). (B) Similarly, when Hs683 cells were cultured in dialyzed media supplemented with 20 mM glutamate, increasing concentrations of JNJ reduced viability with an IC\textsubscript{50} of 182 µM (CI 95% (152-218)). Conversely, when increasing concentrations of JNJ were added to dialyzed media (with no supplemental glutamate and GPT added to enzymatically remove glioma secreted glutamate), non-linear regression did not fit the data. (C) A second non-competitive antagonist, CPCCOEt, also inhibited Hs683 viability when added to dialyzed media supplemented with 20 mM glutamate with an IC\textsubscript{50} of 321 µM (CI 95% (271-381)). When increasing concentrations of CPCCOEt were added to dialyzed media with GPT, non-linear regression did not fit the data. Each data point represents the mean of 3 independent experiments (n) (± SEM) measured in triplicate. The data were analyzed with GraphPad Prism 7.0 using the non-linear regression, variable slope, four parameter equation.
Figure 12. Representative photographs of Hs683 glioma cells prior to MTT assay. 6 days after plating on a 96-well plate and 5 days after subjected to treatment conditions (A) Full media (B) Dialyzed media (C) Dialyzed media supplemented with 20mM glutamate and (D) Full media with 300μM JNJ16259685.
Figure 13. Selective non-competitive antagonists of the mGlu1 receptor dose dependently decrease the viability of all glioma cell lines tested. CPCCOEt and JNJ16259685 blocked 20mM glutamate promotion of cell viability in dialyzed serum in a dose dependent manner in Hs683, A172, and U87 glioma cell lines. Bar graphs represent cellular viability as measured by MTT and normalized to viability in full serum. Each data point represents the mean of 3 independent experiments (n) (± SEM) measured in triplicate. One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01, comparing to viability in dialyzed media + 20 mM glutamate).
Figure 14. The selective mGlu5 receptor antagonist MPEP did not influence glioma cell line viability. Bar graphs represent cell viability measured by the MTT assay and normalized to viability in full serum. MPEP had no significant impact on 20mM glutamate promotion of cell viability in dialyzed serum when administered in doses 10 and 1000-fold higher than MPEP’s reported IC50 at mGlu5 (36nM) 84. Each data point represents the mean of 3 independent experiments (n) (± SEM) measured in triplicate. One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01, compared with viability in dialyzed media + 20 mM glutamate).
Figure 15. mGlu1 receptor shRNA effectively reduced mGlu1a and mGlu1b in Hs683 cells compared with scrambled shRNA. 2% agarose gel containing PCR products. As expected, mGlu1a and mGlu1b amplicons were detected in Hs683 glioma cells transfected with a scrambled shRNA control plasmid. In contrast, mGlu1a and mGlu1b were effectively silenced in Hs683 glioma cells transfected with 4 different mGlu1 targeted shRNA plasmids. GAPDH and mGlu1 products were generated in separate reactions that included equivalent volumes of the same aliquot of cDNA. Photographs were cropped to promote clarity.
Figure 16. GRM1 gene silencing in Hs683 cells significantly reduced glioma cell proliferation. 72 hours after plating on 96 well plates, and 96 hours after transfection with shRNA plasmids, wells containing Hs683 cells treated with mGlu1 shRNA had significantly less nucleic acid content compared with those treated with a scrambled shRNA control or with transfection agent alone (wild type). Relative light units (RLUs) represent measurements of emitted fluorescence by CyQUANT® dye. Each data point represents the mean of 3 independent experiments (n (± SEM), each with three to five replicates. One way ANOVA with Tukey’s Multiple Comparisons Test was utilized to measure the differences between groups at 72 hours (*p<0.01, comparing wild type and control scrambled shRNA to mGlu1 shRNA).
Figure 17. The non-competitive mGlu1 selective antagonist, JNJ16259685, dose dependently inhibits anchorage-independent growth of Hs683 glioma cells. (A) Representative photographs of Hs683 colonies grown in soft agar for 21 days and then stained overnight with nitro-blue tetrazolium chloride. (B) Quantitative analysis of colony formation including the entire well area using Image J. Each bar represents the mean of 3 (100µM) or 4 independent experiments (control, 30, 300µM JNJ) measured in duplicate and normalized to the grand mean of each experiment for analysis and then normalized to the mean of the control group for presentation. Error bars represent the SEM of the independent experiments. The average number of colonies in the control group was 403 (±89) per well and the average size was 29µm² (± 5) per well. One-way ANOVA with Dunnett’s Multiple Comparisons Test was utilized to measure the differences between groups (*p<0.01, comparing relative colony number or average colony size to the mean of the control group).
4.1 Glioma cells release high concentrations of glutamate promoting glioma viability through an autocrine/paracrine loop.

A variety of cancerous cells have been shown to release glutamate including melanoma, breast carcinoma, prostate carcinoma and glioma. It has been widely hypothesized that this released glutamate activates glutamatergic receptors expressed on tumor cells in an autocrine and/or paracrine fashion to stimulate proliferation, migration and survival. Consistent with this hypothesis, all glioma cell lines tested in this study released significant concentrations of glutamate (235-278 µM) measured over a five-day incubation period (Figure 4) and glutamate dose dependently promoted glioma cell viability (Figures 3, 5, and 6). Furthermore, the $EC_{50}$ for glutamate-stimulated viability in Hs683 cells (3.7mM; Figure 3) was consistent with the $EC_{50}$ of glutamate reported to promote viability in mGlu1 positive melanoma cell lines, SK2 ($EC_{50}$ = 4.3 mM) and SK5 ($EC_{50}$ 3.4 mM) 66. The $EC_{50}$ for glutamate-stimulated viability was significantly higher than the measured concentration of released glutamate in Hs683 cells. However, it seems quite likely that our measurement of glutamate release from a monolayer of cultured cells in a well containing 100 µl of media, greatly underestimates glutamate concentrations achieved within solid tumors with significantly higher cell densities. If this assumption is correct, glioma cells in vivo are exposed to concentrations of glutamate much closer the $EC_{50}$ measured in this study. Also, the $EC_{50}$ values reported for glutamate-stimulated
viability in glioma and melanoma cells are higher than the glutamate concentrations observed to promote cell survival in CHO cells heterologously expressing the mGlu1 receptor (EC\textsubscript{50} = 153 µM) and in cerebellar granule neurons (CGN) (EC\textsubscript{50} = 76µM) \textsuperscript{68,70}. While, high mGlu1 receptor expression levels are expected in heterologous systems and have been confirmed in cerebellar granule neurons \textsuperscript{46}, only semi-quantitative methods have been used to confirm mGlu1 expression in glioma (Figure 8)\textsuperscript{31,52,59} and melanoma\textsuperscript{61}. Thus, it is probable that mGlu1 receptor expression is higher in heterologous systems and in CGN compared with the cancer cell lines tested. Agonist potency and efficacy are influenced by mGlu1 receptor expression levels \textsuperscript{90}. Therefore, high levels of mGlu1 receptor expression may account for the increased potency for glutamate-stimulated viability observed in CHO cells stably transfected with the mGlu1 receptor and in CGNs as compared with glioma and melanoma.

4.2 mGlu1a and mGlu1b receptor mRNA expression were observed in all glioma cell lines tested; however, the expression of mGlu1 splice variants 1d, 1f, 1g, and 1h and mGlu1 receptor protein remain unconfirmed.

Using RT-PCR we were able to detect mGlu1 mRNA in five glioma cell lines, validating previous reports of mGlu1 receptor mRNA expression in glioma cells \textsuperscript{31,52,59} (Figure 8). The primers used in this study detected two mGlu1 splice variant transcripts, 1a and 1b, in all glioma cell lines tested (Figure 8) which was consistent with a similar study of mGlu1mRNA expression in melanoma \textsuperscript{77}. In addition to 1a and 1b, four additional splice variants for mGlu1 have been described \textsuperscript{77}. Although the functional differences between these isoforms have not been
thoroughly studied, the length of the C-terminal domain of the receptor is considerably longer in mGlu1a compared with the other splice variants. This had led to speculation that the mGlu1a isoform may participate in more complex scaffold-dependent protein-protein interactions than the other splice variants. Based on the likely functional differences between the isoforms, it would be interesting to determine the relative expression of the 1d, 1f, 1g, and 1h isoforms in glioma in future studies. Assuming one, or more, of these are expressed, it would be important to understand which splice variants increased glioma proliferation.

Although we attempted to test for mGlu1 protein expression, we were unable to obtain a mGlu1 antibody with adequate specificity. Therefore, we cannot say with certainty that the mGlu1 mRNA detected would be ultimately translated into protein. We are aware of only two previous studies that report testing for mGlu1 protein expression in glioma. Aronica and colleagues did not detect mGlu1a protein expression using a western blot technique. However, the researchers only tested one glioma cell line (U-373) and specimens from a single GBM tumor, which limits the ability to generalize these results. In addition, the U-373 cell line used in this study has since been withdrawn from American Type Culture Collection’s (ATCC) cell catalogue after an investigation into the authenticity of the cell line was conducted. Furthermore, the commercial antibody used in the study has been criticized for off-target neuropil labeling in immunohistochemistry assays. Assuming the antibody was indeed specific for the mGlu1a receptor isoform, the possibility that other splice variants were expressed but not detected also cannot be ruled out. In contrast, the mGlu1 receptor protein was detected in the U-87 cell line by Zhang and colleagues using both immunohistochemistry and western blot techniques. However, these results should be interpreted with caution because the authors do
not clarify the origin of the anti-mGlu1 antibody used or provide evidence that controls were conducted to verify antibody specificity.

4.3 Our evidence suggests that ligand bias may exist at the mGlu1 receptor in glioma cells.

It is well established that glutamate activation of the mGlu1 receptor induces a Gαq-protein mediated signaling cascade that activates phospholipase C, stimulating phosphoinositide (PI) hydrolysis and activating protein kinase C. Reported downstream effects include increased intracellular calcium, facilitation of ionotrophic receptor currents⁴⁶, and transient ERK phosphorylation⁶⁸,⁶⁹. Based upon this well-known characterization of mGlu1 receptor signaling, receptor activity has traditionally been stimulated with agonists known to stimulate Gαq signaling and measured using Gαq signaling pathway specific outputs such as intracellular calcium concentration and PI hydrolysis⁶³. However, our laboratory has reported a mechanism by which glutamate-stimulated mGlu1 receptors mediate cell survival that would not be captured by measurement outputs that rely on Gαq signaling⁶³. This mechanism involves a Gαq-independent, β-arrestin dependent, signaling cascade that induces sustained ERK phosphorylation and improved cellular viability⁶⁸. Furthermore, our laboratory has observed evidence of ligand bias at mGlu1 receptors expressed in CHO cells and in cerebellar granule neurons (CGN)⁶⁸,⁷⁰. In both of these models, unbiased ligands, such as glutamate and aspartate, activate either the canonical Gαq pathway or the β-arrestin-dependent pathway and biased ligands such as quisqualate and DHPG only activate the Gαq pathway⁶⁸-⁷⁰.
In the current study, we provide pharmacologic and genetic evidence suggesting that mGlu1 receptor signaling specifically supports glioma proliferation and viability. We were also interested in determining if mGlu1 receptor signaling in glioma cells demonstrated evidence of ligand bias and β-arrestin dependent signaling. Therefore, we measured the ability of both unbiased (glutamate and aspartate) and biased mGlu1 receptor agonists (DHPG and quisqualate) to produce enhanced viability in cells cultured in dialyzed media. We observed that unbiased ligands glutamate (in all cell lines tested) and aspartate (in Hs683 and U87 cells) significantly increased viability compared to cells cultured in dialyzed media (Figure 9). Glutamate acted as a full agonist, while the maximum aspartate dose was limited by solubility constraints at 30mM. Therefore, we were unable to determine if aspartate was a mGlu1 receptor partial agonist with low potency or a full agonist with low potency. Conversely, the Gαq biased ligands, DHPG and quisqualate did not have a significant effect on Hs683, A172, or U87 glioma cell viability when compared with control cells cultured in dialyzed media (Figure 9). This is consistent with the hypothesis that the mGlu1 receptor demonstrates ligand bias in glioma cells and with our laboratory’s previous findings that DHPG and quisqualate do not promote viability in CHO cells stably expressing the mGlu1 receptor, CGN, or in mGlu1 receptor positive melanoma cells.

A previous study conducted in our laboratory used site directed mutagenesis to determine that Thr188 residues were necessary for PI hydrolysis (Gαq signaling), while Arg323 and Lys409 residues were required for β-arrestin dependent signaling. Therefore, a possible explanation for ligand bias in glioma could be that the various agonists have distinct, agonist-specific interactions with the mGlu1 receptor binding domain. However, based on the unique conditions present in glioma, particularly the sustained exposure of mGlu1 receptors to high
concentrations of glutamate, alternative explanations for the apparent ligand bias at the mGlu1 receptor should also be considered.

4.4 Sustained exposure of the mGlu1 receptor to high concentrations of glutamate may promote β-arrestin mediated internalization and pro-survival signaling in glioma.

In this study, we confirmed that mGlu1 receptor mRNA is expressed in Hs683, A172, and U87 glioma cell lines and that these cells release high concentrations of glutamate into the surrounding culture media. Assuming mGlu1 receptor cell surface expression, one would expect these mGlu1 receptors to be continuously exposed to high glutamate concentrations. This prolonged cellular exposure to glutamate may directly influence mGlu1 receptor signaling by stimulating homologous receptor desensitization, a process that includes β-arrestin and dynamin dependent internalization of the receptors. Theoretically, these internalized receptors would not be available at the cell surface to signal through the canonical Gαq signaling cascade. However, they would be available to signal through the Gαq – independent, β-arrestin dependent, signaling pathway of the mGlu1 receptor, ultimately promoting glioma cell viability.

Previous studies of mGlu1 receptor desensitization and internalization in heterologous models support this hypothesis. For example, exposure to 1mM glutamate for 60 minutes reduced mGlu1a cell surface expression in a β-arrestin dependent manner by approximately 70% in HEK-293 cells transiently transfected with mGlu1a. mGlu1 receptor internalization was not reported beyond the 60-minute time point in this study; however, the decline in receptor surface
expression remained linear at 60 minutes. This indicates a further decline would be predicted when glutamate exposure was for an indefinite time period, a condition that would be expected in glioma. It is possible that sustained glutamate exposure at mGlu1 receptors results in a near complete internalization of receptors and a significant decrease in Gαq mediated signaling at the cell surface. For example, one group reported a 30% mGlu1 receptor internalization after a 10 minute, 1mM glutamate, incubation that was associated with a 50% decline in subsequent calcium mobilization. In glioma, where mGlu1 receptors would be exposed to high concentrations of glutamate indefinitely, this effect is likely to be greatly exaggerated. Another independent study confirmed a decline in Gαq mediated responses to quisqualate and DHPG upon mGlu1 receptor internalization. Taken together, these studies suggest that continuous exposure of mGlu1 receptors to high concentrations of glutamate results in progressive β-arrestin mediated internalization of the receptor and decline of Gαq mediated signaling.

To test the hypothesis that glutamate-stimulated viability was dependent on receptor internalization, we used the dynamin inhibitor, dynasore. Dynamin is a cytosolic protein and GTPase that is required for endocytosis of clathrin-coated pits containing mGlu1 receptor: β-arrestin complexes; an event preceding β-arrestin dependent signaling. We found that dynasore dose dependently blocked the positive effect of glutamate on glioma cell line viability in Hs683 cells (Figure 10). At the 100 µM concentration, cell viability was not significantly different than cells cultured in the presence of GPT, indicating a complete block of glutamate-stimulated viability by dynasore. While off target effects of dynasore have been reported, including disruptive effects in the regulation of intracellular cholesterol and in the structure of lipid rafts, we found no change in viability when increasing concentrations of dynasore were
added to dialyzed media (with no supplemental glutamate and GPT added to enzymatically
remove secreted glutamate). This suggests that the observed effect was specifically associated
with blockade of glutamate-stimulated effects and not a non-specific effect. Based on these
results, it is likely that receptor internalization is required in order for glutamate to promote
glioma viability; further supporting our hypothesis that this effect may be β-arrestin dependent in
glioma cells.

Finally, it is important to consider reports suggesting that DHPG and quisqualate could
be less efficacious at inducing receptor internalization compared with glutamate. For example, a
30-minute exposure to 1mM DHPG or 100 µM quisqualate resulted in internalization of
approximately 30% of cell surface receptors; while 1mM glutamate promoted 40% receptor
internalization at 30 minutes and 70% receptor internalization at 60 minutes (60-minute receptor
internalization was not reported for quisqualate or DHPG)\textsuperscript{95}. While it has been shown that
 glutamate-stimulated mGlu1 receptor internalization is mediated by both β-arrestin 1 and β-
arrestin 2\textsuperscript{95}, quisqualate appears to only be internalized by β-arrestin-1\textsuperscript{99}. Based on these
findings, it has been suggested that glutamate and quisqualate may “stabilize different receptor
conformations” that allow for distinct mGlu1 receptor interactions with β-arrestins\textsuperscript{92}. Therefore,
one hypothesis that could explain why unbiased ligands such as glutamate promote glioma cell
viability, while biased ligands DHPG and quisqualate do not, is that quisqualate (and DHPG)
have different receptor internalization kinetics compared with glutamate and/or reduced affinities
for β-arrestin isoforms that are essential for viability effects.
4.5 Glutamate-stimulated glioma cell viability is specifically dependent on mGlu1 receptor signaling.

In this study, we provide pharmacologic and genetic evidence that mGlu1 receptor signaling specifically mediates glutamate-stimulated glioma proliferation and viability. We found that the selective mGlu1 non-competitive antagonist JNJ16259685 (JNJ) dose dependently reduced glutamate-stimulated Hs683 cell viability and report an IC$_{50}$ of 192µM in full media (Figure 11A), and 182µM in dialyzed media supplemented with glutamate (Figure 11B). These values are consistent with the reported IC$_{50}$ for inhibition of viability in mGlu1 receptor positive melanoma cell lines SK2 (109µM) and SK5 (105µM) $^{66}$ and in cerebellar granule neurons (19.8µM) $^{70}$. Similarly, we found that another selective mGlu1 receptor non-competitive antagonist, CPCCOEt, also dose dependently reduced glutamate-stimulated Hs683 cell viability, with an IC$_{50}$ of 321µM (Figure 11C). This result is similar to the reported CPCCOEt IC$_{50}$ for viability in cerebellar granule neurons (123µM) $^{70}$. The IC$_{50}$ values for viability are considerably higher than the IC$_{50}$ values reported for inhibition of Gαq mGlu1 signaling, which are 1.2-19 nM for JNJ $^{100}$ and 6.5µM for CPCCOEt $^{101}$. It has been previously suggested that the difference in IC$_{50}$ values for the Gαq mGlu1 signaling effect and the glutamate-stimulated viability effect may reflect “biased antagonism;” where the noncompetitive antagonists JNJ and CPCCOEt have an increased potency for receptor conformations that inhibit Gαq signaling compared with receptor conformations that inhibit glutamate-dependent viability effects $^{70}$. Another proposed explanation for the differences in antagonist potency could be that receptor reserve exists for β-arrestin dependent pro-viability signaling $^{68}$. If this were true, it would explain the need for a
higher dose of antagonist to block the viability effect. Furthermore, a third possibility exists in glioma, where sustained elevations in local glutamate concentration at mGlu1 receptors would be expected to induce significant β-arrestin dependent receptor internalization. In this context, it is likely that higher concentrations of antagonist are required to overcome the cell membrane barrier to reach internalized mGlu1 receptors.

The possibility that JNJ and CPCCOEt could be having non-specific effects at other glutamatergic receptors must also be considered. It is conceivable that higher JNJ and CPCCOEt doses could block the other group I receptor, mGlu5. However, if mGlu5 receptors were indeed mediating this viability effect, we would expect to see an effect with MPEP, which is a highly selective and potent mGlu5 receptor antagonist. However, even when we tested MPEP at 30µM, which is 1000-fold higher than the reported EC$_{50}$ of 36nM$^{84}$, we did not see any effect on viability (Figure 14). Therefore, glutamate-stimulated viability is unlikely to be mediated through mGlu5 receptor signaling.

As discussed in the introduction, there is substantial evidence that both ionotropic and group II metabotropic glutamate receptors promote glioma proliferation, migration, and survival$^{24,32-37}$. Therefore, it is also possible that the viability effects we are observing with higher doses of JNJ and CPCCOEt may reflect activity at those receptors. However, when shRNA targeted against GRM1 was transfected into Hs683 cells, we measured significantly reduced glioma cell proliferation (Figure 16) compared with scrambled shRNA controls. This validates our pharmacological data and suggests that the mGlu1 receptor is specifically involved in glioma proliferation. While transfection efficiencies for the mGlu1 shRNA and scrambled shRNA control plasmids were robust and roughly equivalent (as measured by GFP signal), they were not
100%. Therefore, it is unclear if the small amount of proliferation noted between 24 and 72 hours in the shRNA treated cells (Figure 16) represents the proliferation of un-transfected cells exclusively or if it reflects a decreased proliferation rate in cells where the mGlu1 receptor was knocked down. It is interesting to note that we were unable to establish a mass stable cell line using the puromycin resistance gene in the shRNA plasmid. This is consistent with the hypothesis that the presence of the mGlu1 receptor is necessary for sustained viability and cellular proliferation.

4.6 The mGlu1 antagonist, JNJ, dose dependently reduced anchorage-independent growth in Hs683 cells.

Anchorage independent growth is a widely used, highly reliable test of malignant transformation. The assay demonstrates the ability of cells to proliferate without binding to an extracellular matrix, predicting an in vivo capability to form tumors and to metastasize. When we cultured Hs683 cells in soft agar, the cells exhibited significant anchorage-independent growth with an average colony number of 403 (± 89) per well and average colony size of 29µm² (± 5) (Figure 17A and B). However, as increasing concentrations of JNJ were added to the soft agar, both colony size and colony number were dose dependently reduced, indicating a conversion to an anchorage-dependent phenotype. It was particularly interesting to note that a trend towards decreased colony size was observed at 30µM JNJ, and a significant difference in both colony size and colony number were measured at the 100µM concentration (Figure 17A and B). Both of these JNJ concentrations are substantially lower than the measured IC₅₀ for
inhibition of glutamate-stimulated viability in cell culture. As shown in Figures 11A and 11B, 100µM JNJ did not significantly inhibit the viability of Hs683 cells. Therefore, the reduction in colony number and size observed in the soft agar assay (Figure 17A and 17B) at 100µM JNJ is likely to specifically reflect an inhibition of anchorage dependent growth, rather than a reduction in proliferation or cell viability in general. These results suggest that noncompetitive antagonism of the mGlu1 receptor with JNJ may decrease the tumorigenic and metastatic potential of glioma cells in vivo. Assuming the JNJ EC_{50} for prevention of anchorage-independent growth to be approximately 50µM (Figure 17B), these results also suggest that the effective dose to reduce tumor growth in vivo may be nearly four-fold less than doses predicted by our cell culture model (Figure 11A-C).

4.7 A previous report supports the hypothesis that the mGlu1 receptor drives glioma cell proliferation and survival; but it should be interpreted with caution.

While several independent groups have reported aberrant expression of mGlu1 mRNA in glioma, we are aware of only one other study that focused on mGlu1 receptor signaling in glioma. This study used siRNA and pharmacologic agents to inhibit mGlu1 signaling in U87 GBM cells in cell culture and in athymic mice xenograft models. The researchers report decreased viability, increased apoptosis, decreased migration, decreased tumor growth, and decreased PI3K/Akt/mTOR activation with pharmacologic and genetic mGlu1 receptor inhibition. The overall conclusion of the study, that the mGlu1 receptor drives malignant tumor growth, migration and survival, is consistent with our findings. However, there are several
limitations in study design and internal inconsistencies that reduce the strength of the evidence presented. First, the authors state that they used two “selective non-competitive mGluR1 antagonists” riluzole and Bay36-7620 to demonstrate pharmacologic antagonism of the mGlu1 receptor. While Bay36-7620 is appropriately categorized as a potent, selective non-competitive mGlu1 receptor antagonist, riluzole is more accurately categorized as an inhibitor of glutamate release and GABA uptake with antagonist activity at NMDA receptors. Therefore, the riluzole data could reflect action at any glutamatergic receptor and cannot be interpreted as evidence of specific mGlu1 receptor activity. Furthermore, the authors report a finding that is difficult to interpret; they state that when they knocked down mGlu1 with siRNA or when they inhibited mGlu1 receptor activity with non-competitive antagonists, they observed a decrease in viability that could be reversed with 10µM quisqualate. Not only does this contradict our finding that quisqualate concentrations as high as 300µM do not promote glioma cell line viability (Figure 9), but it is also highly unlikely that the viability effects could be attributed to a “rescue” of mGlu1 receptor function, as noncompetitive antagonist effects and siRNA inhibition would not be expected to reversed by an agonist.

Finally, an additional limitation of this study was that they only used the U87 cell line. A recent analysis of this widely used cell line using DNA fingerprinting and genotyping, determined that the original U87 cell line and tumor were different than the U87 cell line available for purchase from ATCC (also the origin of the U87 cells used in this study). The authors then compared the ATCC cell line for transcriptional similarity to a data base of over a thousand cell lines and concluded that the ATCC U87 cell line was highly likely to be a GBM of unknown patient origin. Therefore, while it remains appropriate to consider ATCC U87 cells
to be representative of GBM, this finding emphasizes the importance of working with multiple cell lines and the need to ultimately confirm our findings in Hs683, U87, and A172 cell lines with primary cultures from original tumor specimens.

4.8 The mGlu1 receptor exhibits characteristics of a proto-oncogene.

A common finding in cancer genetics is the “selective re-expression” \(^{106}\) of genes that regulate early growth and development \(^{107}\). The mGlu1 receptor has been associated with neurogenesis, including the enhancement of neural progenitor cell proliferation \(^{108-110}\). Furthermore, the mGlu1 receptor is expressed and appears to be an important regulator of oxidative stress in immature oligodendrocytes, with expression levels dropping dramatically as the oligodendrocyte matures \(^{54,111}\). In the present study, we detected mGlu1 mRNA in the Hs683 glioma cell line, which is classified as an oligodendroglioma with a GBM phenotype \(^{71,112}\). We also detected mGlu1 mRNA expression in several cell lines classified as high grade astrocytoma or GBM (See Figure 8; U87, A172, and U118 cell lines). The consistent expression of mGlu1 receptor mRNA in high grade astrocytoma \(^{31,52,59}\) is somewhat surprising because the mGlu1 receptor has not been detected in normal astrocytes and has only been found in spinal cord astrocytes in pathological contexts such as traumatic injury or ALS \(^{53,56-58}\). This finding parallels what has been seen in melanoma, where mGlu1 receptors are not detected in melanocytes but are detected in the malignant phenotype, melanoma \(^{64,65}\). As discussed in the introduction, there is substantial evidence to suggest that the mGlu1 receptor acts as a proto-oncogene when aberrantly expressed in melanoma. Among the most convincing evidence, is the report that mGlu1 cDNA
transfection into melanocytes transforms the cells to a melanoma phenotype which can be subsequently reversed with knock-down of the receptor. In the present study, we provide evidence that the mGlu1 receptor may play a similar role in glioma. For example, we demonstrate that glioma cell viability, like melanoma cell viability, is dependent on glutamate (Figures 3, 5 and 6), and that blockade of the mGlu1 receptor signaling with mGlu1 receptor selective non-competitive antagonists (Figures 11-13) and with genetic silencing (Figure 16) significantly reduces glutamate-stimulated glioma cell viability and proliferation. In addition, we demonstrate that the non-competitive selective mGlu1 receptor antagonist, JNJ16259685, can convert Hs683 cells grown in soft agar from an anchorage-independent phenotype to an anchorage-dependent phenotype; predicting that Hs683 cells treated with this antagonist will exhibit a less metastatic and tumorigenic characteristics in vivo (Figure 17). Taken together, these results strongly suggest that the mGlu1 receptor may act as a proto-oncogene in glioma by promoting dysregulated proliferation and survival of Hs683, U87, and A172 cells in a manner similar to the role of the mGlu1 receptor in melanoma. This effect may be the result of “selective re-expression” of GRM1, the mGlu1 receptor gene, which in normal developmental physiology, drives the proliferation of neural progenitor cells and promotes the survival of immature oligodendrocytes under conditions of oxidative stress. Thus, inhibition of mGlu1 receptor signaling may offer an alternative strategy for treating glioma. Future testing in translational models will be important to determine if mGlu1 receptor signaling can provide an opportunity to diversify GBM treatment in an effort to combat treatment resistance and improve the prognosis of this devastating disease.
CHAPTER 5
FUTURE DIRECTIONS

5.1 The molecular mechanisms underlying mGlu1 receptor mediated glioma proliferation and survival warrant future investigation.

The results presented in this dissertation demonstrate that mGlu1 receptor mRNA is expressed in glioma and that the mGlu1 receptor specifically promotes glioma cell proliferation, viability and anchorage independent growth. Therefore, it appears that the mGlu1 receptor could be an effective pharmacologic target for treating glioma as well as other mGlu1 receptor positive neoplasms. However, the intracellular signaling mechanism responsible for these effects remains unknown. An investigation into the precise mechanism for these effects is important because it may reveal additional down-stream targets that selectively block glioma viability and/or possible mechanisms of treatment resistance. Data presented in this thesis support the hypothesis that a β-arrestin dependent signaling cascade may be involved in glutamate-stimulated viability in glioma cells and that ligand bias may exist at mGlu1 receptors expressed in these cells. For example, we observed complete blockade of glutamate-stimulated viability with the dynamin inhibitor, dynasore, indicating that receptor internalization was required for pro-survival mGlu1 receptor signaling. We also found that unbiased agonists glutamate and aspartate promoted viability, but quisqualate and DHPG did not, a finding that has been consistent in every model of mGlu1 receptor expression tested by our laboratory, including CHO cells heterologously
expressing the mGlu1a receptor, cerebellar granule neurons, and in mGlu1 positive melanoma cells.63,68,70

The molecular mechanism underlying protective signaling and ligand bias remains unclear. We have identified three potential hypotheses to explain these effects. The first is the possibility that biased and unbiased ligands bind to the mGlu1 receptor at different sites and/or stabilize different receptor conformations. This hypothesis is supported by a previous study conducted in our laboratory that determined that Thr188 residues were necessary for PI hydrolysis (Gq signaling) while Arg323 and Lys409 residues were required for β-arrestin dependent signaling.69 A second hypothesis is that unbiased agonists have increased affinities and/or potencies for inducing β-arrestin internalization compared with biased ligands. There is also evidence suggesting that this could be true as glutamate promotes mGlu1 receptor binding to either β-arrestin 1 and β-arrestin 2, while quisqualate only promotes mGlu1 receptor binding to β-arrestin 1.95,99 In future studies, it would be interesting to determine relative β-arrestin isoform expression in glioma cells and to measure glutamate-stimulated glioma viability with selective knock down of the various β-arrestin isoforms. These experiments could confirm that the glutamate-stimulated viability effect is dependent on β-arrestin and identify the specific β-arrestin isoform(s) involved. If it is determined that β-arrestin 2 selectively mediates the pro-signaling pathway, that could explain why quisqualate is ineffective at promoting pro-survival signaling. We also present a third possibility below; that mGlu1 receptor dimerization status may influence the signaling pathway activated by the various mGlu1 receptor agonists.
5.2 The prevailing theory describing mGlu1 receptor structure-function relationships is based on the assumption that mGlu1 receptors can only signal as homodimers.

Metabotropic glutamate receptors and the GABA B receptor are members of the group C GPCR family. Class C GPCRs share significant sequence homology and structural similarity, particularly in the orthosteric ligand-binding domain (LBD) and the dimerization interface. Existing evidence suggests mGlu1 exclusively forms homodimers. The dimeric structure is stabilized by a non-critical covalent disulfide bond at Cys, by a direct interaction of large hydrophobic regions within each protomers LBD, and by a recently identified TMD dimeric interface mediated by six cholesterol molecules. It is likely the direct interaction of the hydrophobic LBRs is critical because GABA B receptor interface disruption resulted in a loss of function. Dimerization of the mGlu1 receptor is assumed to be essential for signal transduction. This was initially suggested when the crystal structure of the mGlu1 receptor’s LBD was published. A comparative analysis between the ligand free and glutamate bound conformations revealed a significant shift in the orientation of the two protomers relative to each other. It was hypothesized that ligand binding at the orthosteric site induces dimeric reorganization which then produces a conformational change in the transmembrane domain (TMD) enabling G protein-mediated cell signaling. Subsequently, a number of functional studies using site directed mutagenesis and Förster resonance energy transfer have supported the hypothesis that this dimer rearrangement is a prerequisite for canonical G protein coupled signaling.
5.3 Studies relying exclusively on measurement of canonical G-protein signaling cascade activity (such as PI hydrolysis) provide an incomplete profile of mGlu1 receptor activation.

CHO cells transfected with wild-type mGlu1 produce robust PI hydrolysis in response to glutamate. However, melanoma cell lines expressing the mGlu1 receptor produce no detectable PI hydrolysis. Based upon these data, it would appear that the mGlu1 receptor expressed in melanoma was nonfunctional. However, as we discussed in detail in the introduction, studies measuring melanoma cell viability indicate that these mGlu1 receptors have a significant influence on cell survival. This example highlights the importance of considering each mGlu1 receptor signal transduction pathway independently when making conclusions regarding receptor function. In future studies, it would be interesting to determine if glioma cells also fail to produce detectable PI hydrolysis. Based upon our theory that the majority of mGlu1 receptors expressed on glioma cells would be internalized secondary to homologous desensitization, we would predict a similar lack of detectable PI hydrolysis in glioma. Future studies should therefore, include a control experiment where the glioma cells are incubated in the presence of GPT to reduce homologous desensitization and determine if PI hydrolysis can be restored.

5.4 In contrast to Gαq activation, β-arrestin dependent signaling may not require dimerized mGlu1 receptors.

mGlu1 receptor protein immunoblots routinely show bands at both the monomeric and
dimeric molecular weights. The presence of both molecular species is typically attributed to an artifact of experimental conditions in which SDS incompletely disrupts non-covalent interactions and reducing agents incompletely disrupt disulfide bonds. However, no studies have conclusively demonstrated that natively expressed mGlu1 receptors must exclusively signal in the dimeric form. Monomeric forms of some receptors such as neurotrophin receptor p75 have been shown have distinct signaling profiles. Evidence also demonstrates that GPCRs are capable of signaling as monomers. For example, β-adrenergic GPCR receptors integrated into spatially constrained reconstituted high-density lipoproteins could activate Gαs as monomeric entities. In addition, a bioluminescence-resonance energy transfer study indicates that when an agonist stimulates cell-surface β-adrenergic receptor multimers, they become internalized in a β-arrestin manner and disassociate. This supports the possibility that β-arrestin dependent signaling could be transduced by a monomeric GPCR.

5.5 “Forced” monomer mGlu1 receptors have been constructed to test the hypothesis that protective β-arrestin dependent signaling could be produced by mGlu1 receptor monomers.

We developed forced monomer constructs using the method reported to disrupt GABA B receptor dimerization. mGlu1a receptors were mutated to express N-glycosylation sites (through introduction of a three residue N-glycosylation consensus sequence NXS where X is any residue except proline) at the LBD dimer interface that serve to physically occlude dimerization through addition of a “glycan-wedge.” Additionally, a QXS control mutation,
which would not result in N-glycosylation, was also designed to account for any disruptions made by the mutation alone. These constructs (Table 1 and Figure 18) were introduced into both N-terminal FLAG and myc epitope-tagged mGlu1 receptors and cloned into a pIRES/ACGFP1 vector for subsequent transfection into CHO cells (see Methods).

5.6 As predicted, mGlu1a receptors forced into the monomeric conformation through dimer interface glycosylation do not signal through the Gaq pathway.

We conducted preliminary experiments (n=2) using CHO cells transfected with either wild type constructs, the glycosylated forced monomer constructs (N166) or the control mutant constructs (Q166) to test receptor signaling through the Gaq pathway. The graphs in Figure 19 demonstrate that PI hydrolysis was stimulated by glutamate 2 to 3-fold in cells transfected with the wild-type construct and the Q166 control mutant, but the N166 mutant was insensitive to glutamate. The glutamate-stimulated PI hydrolysis in the wild-type and Q166 control mutant cells was completely antagonized by JNJ16259685. The pattern of response to PI hydrolysis in N166 cells was indistinguishable from untransfected CHO cell responses, while the pattern of responses in Q166 was nearly identical to the pattern of response seen in WT mGlu1 receptor transfected CHO cells. These results are consistent with our hypothesis that the N166 mutant cannot dimerize and thus, cannot stimulate PI hydrolysis. The control mutant (Q166), showing no effect is consistent with the mutation itself having no effect.

In N177 and Q177 mutants (Figure 19, middle panel), a quantitatively similar result was obtained, but the level of stimulation appeared to be diminished in the Q177 mutant, suggesting
that the mutation itself was having an effect. In N170 mutants (n=1) (Figure 19, bottom panel), introduction of the glycosylation site did not impair PI hydrolysis. Taken together, these experiments suggest that dimerization is indeed required for mGlu1 receptor mediated Gαq activity. In future experiments, it would be critical to verify cell surface expression of the N166 and N177 mutants using antibodies targeting the myc and FLAG tag epitopes and to verify with co-immunoprecipitation experiments that the N166 mutant does not dimerize and the Q166 does. Also, as a test of our hypothesis, it would be important to investigate if overexpression of the N166 mutant, as well as the Q166 mutant, both stimulate cell proliferation and promote glutamate-stimulated viability.

5.7 The mGlu1 receptor as a pharmacologic target in glioma treatment.

Ionotropic glutamate receptor function is essential for fast synaptic transmission, while the role of metabotropic receptors in normal physiology is more modulatory in nature\textsuperscript{126}. Therefore, it has been widely suggested that metabotropic glutamate receptors may be superior drug targets because they would be expected to have less dramatic effects on normal central nervous system function\textsuperscript{32,126}. Ligand bias at the mGlu1 receptor may offer an additional layer of selectivity as 1) the structure-function relationships underlying mGlu1 receptor intracellular signaling may be discrete between the different pathways and 2) it may be possible to pharmacologically inhibit one signaling pathway while leaving another intact. This could have important clinical implications. For example, it is likely that mGlu1 receptor mediated Gαq activation of protein kinases and increases in intracellular calcium concentrations play important roles in normal
Therefore, it would be ideal to develop highly targeted therapeutics that reduce glioma proliferation and viability through selective antagonism of β-arrestin signaling at the mGlu1 receptor while maintaining normal Gαq mediated signaling.

Finally, and most importantly, future studies should be conducted to confirm that the mGlu1 receptor acts as a proto-oncogene in alternative experimental models. One potential experiment would be to use viral transduction to introduce mGlu1 receptor cDNA into normal astrocytes and observe for signs of cellular transformation such as anchorage independent growth and the capacity of cell xenografts to form tumors in murine models, similar to previous research conducted in melanocytes. Our results should also be replicated in primary cultures from confirmed GBM surgical specimens and in animal models to determine if mGlu1 antagonism is relevant in an in vivo context. Fortunately, JNJ16259685 effectively crosses the blood brain barrier and will be useful in completing these studies.

In summary, this thesis used three glioma cell lines Hs683, A172, and U87 to confirm that mGlu1 receptor mRNA was widely expressed in glioma cell lines and that glioma cell viability was dependent on the continuous presence of glutamate. This thesis also provides pharmacologic and genetic evidence suggesting that mGlu1 receptor signaling specifically supports glioma proliferation and viability and that non-competitive antagonism of the mGlu1 receptor prevents significant anchorage independent growth of Hs683 glioma cells. Taken together, these results suggest that the mGlu1 receptor may be a viable drug target in the treatment of glioma, a disease with a poor prognosis and limited treatment options.
Table 1. Forced-Monomer Point Mutations

<table>
<thead>
<tr>
<th>Reference Residue</th>
<th>S166</th>
<th>Q170</th>
<th>L177</th>
</tr>
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<tbody>
<tr>
<td>Wild-Type Sequence</td>
<td>SVA</td>
<td>QVQ</td>
<td>LFD</td>
</tr>
<tr>
<td>Forced Monomer Sequence</td>
<td>NVS</td>
<td>NVS</td>
<td>NFS</td>
</tr>
<tr>
<td>Control Mutant Sequence</td>
<td>QVS</td>
<td>QVS</td>
<td>QFS</td>
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</tbody>
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Table 1. Forced-Monomer Point Mutations used to produce glycosylated “forced monomer” and control mutant mGlu1a receptors.
Figure 18. Molecular modeling of the mGlu1 dimer interface with spherical representations of putatively glycosylated residues. (A) N166 and (B) N177 and glutamate bound at the orthosteric binding site.
Figure 19. Monomeric mGlu1a receptors (N166 and N177) do not signal through PI hydrolysis when stimulated by glutamate. However, the control dimeric mutant receptors (Q166 and Q177) retain Gαq function as evidenced by PI hydrolysis similar to wild type FLAG mGlu1. The N170 mutation had no effect. Graphs representing PI hydrolysis.
normalized to fold change over basal measurement signal in untransfected CHO cells, CHO cells transfected with FLAG tagged mGlu1 receptor, putative forced monomers (N166, N177, and N170), and null mutations (Q166, Q177, and Q170). N= asparagine, Q= glutamine. Numbers (i.e. 166) represent the residue at which the putative N-glycosylation sites or control mutations were introduced. Each data point represents the mean of 2 independent experiments (166 and 177) and 1 experiment (170) (± SD) measured in duplicate. Two-way ANOVA with Tukey’s multiple comparisons test was utilized to measure the differences between groups (*= p< 0.05).
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