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The Role of Metabotropic Glutamate Receptor 1 Dependent Signaling in Glioma Viability*

Carrie Bowman Dalley, Barbara Wroblewska, Barry B. Wolfe, and Jarda T. Wroblewski‡

The Wroblewski Laboratory, Department of Pharmacology and Physiology (C.B.D., B.W., B.B.W., J.T.W.) and School of Nursing and Health Studies (C.B.D.), Georgetown University Medical Center, Washington DC 20057

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Corresponding Author: Carrie Bowman Dalley

Address: St. Mary's Hall Rm. 430, 3700 Reservoir Rd. NW, Washington DC, 20057

Tel.: 202-687-2189

Fax: 202-687-2323

Email address: ccb23@georgetown.edu

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Nonstandard Abbreviations:

cDNA (Complementary deoxyribonucleic acid)

CHO (Chinese hamster ovary)

CGN (Cerebellar granule neurons)

CPCCOEt (7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester)

DMEM (Dulbecco's modified eagle medium)

DHPG (3,5-Dihydroxyphenylglycine)

DMSO (Dimethyl sulfoxide)

EAAT1/2 (Sodium and potassium-dependent excitatory amino acid transporters)

eGFP (Enhanced green fluorescent protein)

ERK (Extracellular-signal regulated kinase)

FBS (Fetal bovine serum)

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)

GBM (Glioblastoma multiforme)

GPT (Glutamate pyruvate transaminase)

GRM1 (Glutamate metabotropic receptor 1 gene)

JNJ16259685 ((3,4-dihydro-2H-pyrano[2,3]b quinolin-7-yl) (cis-4-methoxycyclohexyl)-methanone)

mGluR1 (Metabotropic glutamate 1 receptor)

mGluR5 (Metabotropic glutamate 5 receptor)

MPEP (2-Methyl-6-(phenylethynyl)pyridine)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

PBS (Phosphate-buffered saline)

PI (Phosphoinositide)

RLUs (Relative light units)

shRNA (Small/short hairpin ribonucleic acid)

siRNA (Small/short inhibitory ribonucleic acid)

SK2 (SK-MEL-2)

SK5 (SK-MEK-5)

System x_c^- (Cystine/glutamate antiporter)

WHO (World Health Organization)

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. Based on reports of aberrant expression of mGluR1 mRNA in glioma, evidence that melanoma growth is directly influenced by mGluR1, and characterization of β-arrestin dependent pro-survival signaling by this receptor, this study investigates the hypothesis that glioma cell lines aberrantly express mGluR1 and depend on mGluR1 mediated signaling to maintain viability and proliferation. Three glioma cell lines (Hs683, A172 and U87) were tested to confirm mGluR1 mRNA expression and the dependence of glioma cell viability on glutamate. Pharmacologic and genetic evidence is presented that suggests mGluR1 signaling specifically supports glioma proliferation and viability. For example, selective non-competitive antagonists of mGluR1, CPCCOEt and JNJ16259685, decreased the viability of these cells in a dose dependent manner and GRM1 silencing significantly reduced glioma cell proliferation. Also, results of an anchorage-independent growth assay suggest that noncompetitive antagonism of mGluR1 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 mGluR1 expressed in these cells. Taken together, the results strongly suggest that mGluR1 may act as a proto-oncogene in glioma and be a viable drug target in glioma treatment.

Introduction

Gliomas are 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 (Ostrom, et al., 2014; Robert and Sontheimer, 2014). The WHO grades gliomas (I-IV) based on malignant characteristics such as the degree of atypical morphology, mitotic activity, vascularization, and necrotic area (Louis, et al., 2007). 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 (Zinn, et al., 2013). The current standard of care for GBM involves radiation therapy, surgical resection, and temozolomide (Gilbert, et al., 2014; Lacroix, et al., 2001; Stupp, et al., 2009). However, even with these treatments, GBM survival rates are reported to be only 12-16 months following diagnosis (Robert and Sontheimer, 2014; Roth and Weller, 2014), and GBMs account for the highest number of years of life lost among malignant tumors (Schwartzbaum, et al., 2006). Therefore, there is an urgent need to identify more effective pharmacological targets.

One of the largest obstacles in achieving this goal has been the rapid development of resistance to various glioma treatments. For example, it appears when a single component of a tumorigenic signaling pathway (e.g. epidermal growth factor receptor or vascular endothelial growth factor) is pharmacologically inhibited or targeted with an immunotherapy strategy, compensatory responses occur within that signaling axis to sustain tumor viability and/or inhibition confers a selective advantage to cell populations capable of maintaining viability through alternative cell signaling pathways (Roth and Weller, 2014; Soda, et al., 2013; Malkki, 2016). Thus, it is likely GBM treatment may require concurrent therapies targeting relatively

independent mechanisms of tumor proliferation, survival, and migration.

Glutamate is considered the primary excitatory neurotransmitter in the brain and an important mediator of proliferation, migration, and survival during neuronal development (Rzeski, et al., 2001). The typical extracellular glutamate concentration in the brain is 1-3 μM (de Groot and Sontheimer, 2011). However, following synaptic glutamate release, glutamate concentrations are estimated to reach 1mM at the synapse and up to 190μM in perisynaptic regions (Dzubay and Jahr, 1999). Glioma cells *in vitro* and GBM tumors *in vivo* have been reported to release high concentrations of glutamate (Robert and Sontheimer, 2014). This finding is attributed to inhibition of reuptake due to downregulation of EAAT1/2 expression and overexpression of system x_c^- leading to increased glutamate secretion into the extracellular space (Robert and Sontheimer, 2014; Thomas, et al., 2015). Gliomas have a distinct survival advantage in this environment; neuronal glutamate receptors become chronically overstimulated, resulting in excitotoxicity and neuronal death, while glioma cells thrive in high concentrations of glutamate (de Groot and Sontheimer, 2011; Ye and Sontheimer, 1999). Furthermore, the death of neurons provides space for glioma tumor growth within the constrained cranial compartment (Robert and Sontheimer, 2014).

Group I metabotropic receptors (mGluR1 and mGluR5) have been associated with several types of cancer (Willard and Koochekpour, 2013). For example, aberrant expression of mGluR1 in breast cancer increased malignant characteristics including proliferation, anchorage independent growth, and invasiveness (Speyer, et al., 2008; Banda, et al., 2014). Furthermore, studies in our laboratory (Gelb, et al., 2014: Gelb, et al., 2015a; Gelb, et al., 2015b) and others (Marín, et al., 2006; Namkoong, et al., 2007; Shin, et al., 2008) have demonstrated that mGluR1 functions as a proto-oncogene in melanoma. However, compared with ionotropic and group II

metabotropic glutamate receptors, mGluR1 has received little attention in glioma research. Several independent studies suggest that mGluR1 is expressed in malignant glioma (Stepulak, et al., 2009; Brocke, et al., 2010; Parsons, et al., 2008). However, mGluR1 mRNA or protein, has not been detected in normal brain astrocytes (Aronica, et al., 2003; D'Antoni, et al., 2008) or mature oligodendrocytes (D'Antoni, et al., 2008). This observation is particularly interesting when one considers the likely role of mGluR1 in driving tumor growth in malignant melanoma and triple-negative breast carcinoma. For example, mGluR1 cDNA cloned into melanocytes and then allografted into a mouse model, results in the formation of invasive, highly vascularized tumors (Shin, et al., 2008). This tumorigenic phenotype was significantly reduced with siRNA treatment, indicating that mGluR1 expression was critical for maintenance of the malignant transformation (Shin, et al., 2008).

Our laboratory has reported a mechanism by which glutamate-stimulated mGluR1 mediates cell survival and proliferation. This mechanism involves ligand bias and a Gαq independent, β-arrestin dependent signaling cascade that induces sustained ERK phosphorylation and subsequent improved viability in serum-deprived cell cultures (Emery, et al., 2010). These effects have been confirmed in CHO cells stably expressing mGluR1(Emery, et al., 2012), and have been supported by results in cerebellar granule neurons (Hathaway, et al., 2015), and in mGluR1 positive melanoma cells lines (Gelb, et al., 2015b). Our laboratory's findings indicate that this pro-survival signaling pathway may be active in a variety of mGluR1 expressing cell types and may reveal the receptor to be a novel drug target against multiple types of malignant growth, including glioma.

Therefore, based on reports of aberrant expression of mGluR1 mRNA in glioma, accumulating evidence that melanoma growth is directly influenced by mGluR1 signaling, and

characterization of β-arrestin dependent pro-survival signaling by this receptor, we investigated the possibility that mGluR1 influences 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 cell lines served as models for GBM (Le Mercier, et al., 2009; Duffau, 2013; Camby, et al., 2000; Giard, et al., 1973; Maglott, et al., 2006).

Material and Methods

Materials

Glioma cell lines (H4, Hs683, U87, A172, and U118) were provided by the Lombardi Comprehensive Cancer Center Tissue Culture Shared Resource at Georgetown University (Washington, DC). 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 (PubChem CID:5717066), and MPEP were purchased from Tocris Bioscience (Bristol, United Kingdom).

Cell culture

All glioma cell lines were cultured in DMEM media supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), 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 (mol. wt. <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 5% CO_2. CHO cells were cultured under the same conditions with proline supplementation (4.5g/500ml) of the media.

Viability assays (MTT and Calcein AM)

Cells were plated in 100 µl of full media on a 96 well plate: 7000 cells per well for U87 cells, 9000 cells per well for Hs683 and A172 cells, respectively. The following day, the drugs were added. Five days later, the ability of the cells to metabolize MTT (thiazolyl blue tetrazolium) (Life Technologies, Carlsbad, CA) was measured according to the manufacturer instructions on an Envision plate reader (Perkin-Elmer, Waltham, MA). For Calcien AM determination, the cells were incubated as above. Five days after drug application, Calcein AM was added. After a 30-minute incubation period at 37 °C, fluorescence was quantified on the plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Measurement of glutamate concentration

The concentration of glutamate released by the cells was measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Life Technologies) without the amplification step. U87 (7000 cells/well), A172, and Hs683 (each 9000 cells/well) cells were plated on 96 well plates in 100 µl of full media. Twenty-four hours later, the cells were washed with PBS twice and 100 µl per well of media containing dialyzed serum (lacking glutamate) was applied. The media was collected on days 1, 3, and 5 following the media change and the glutamate concentration was quantified by Amplex Red fluorescence as measured at 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 (Supplemental Figure 1).

Glutamate Pyruvate Transaminase (GPT)

Unless otherwise indicated, GPT was applied to cell cultures at a concentration of 35 μg/ml with 10 mM pyruvate, 24 hours after plating. After a five-day incubation period, an MTT assay was used (as above) to quantify the GPT effect.

Detection of mGluR1 mRNA

Total RNA was extracted from glioma cells cultured on 60 mm 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 mGluR1 expression in the various samples using the Phusion High-Fidelity DNA Polymerase Kit and protocol (New England Biolabs, Ipswich, MA). mGluR1 primers were designed to detect mGluR1a (amplicon 650 base pairs) and mGluR1b (amplicon 735 base pairs) as described previously (DiRaddo, et al., 2013). Briefly, the mGluR1 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 mGluR1b contains an 85- base pair exon (IXC) between exons VIII and X that is absent in mGluR1a mRNA. The primer sequences used were forward 2661: ATGCCCATTCTGTCCTACCCAAGT and reverse 3286 GCTCTGGCAAGAGCCTGACCTTTTC. A CHO cell line stably expressing human mGluR1a was used as a positive control. GAPDH detection was used as an internal standard.

mGluR1 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 (#CSHCTR001-CU6) while the other four contained shRNA sequences targeting GRM1 as follows: (starting base listed) 722 (cacgttggataagatcaac), 1507 (aggtcaggtcatttgatga), 1978 (gagtgctgaacattgatga), and 2690 (ggaagtctaccttatctgc) (#HSH008436- (1-4)-CU6). Glioma cells were plated to achieve approximately 80% confluency on 60 mm dishes and incubated overnight at 37°C and 5% CO_2. The following day, the cells were transfected with either a combination of the four plasmids targeting mGluR1 mRNA (total 5 μg cDNA), the plasmid containing scrambled shRNA (5 μg cDNA), or treated with transfection agent only (vehicle). Lipofectamine 3000 Reagent was used following the manufacturer suggested siRNA transfection protocol.

Proliferation assay (CyQUANT Cell Proliferation Kit)

One day after transfection with mGluR1 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 5% CO_2. The samples were collected at 24, 48, and 72 hours after plating, treated according to the manufacturer suggestion and 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 (Supplemental Figure 2).

Anchorage-independent growth

Anchorage- independent growth was measured using the Soft Agar Colony Formation Assay described by Borowicz and colleagues (Borowicz, et al., 2014). In brief, 1.5 ml of 0.5% noble agar in DMEM media containing either 1% DMSO, or 30 μM, 100 μM, or 300 μM JNJ16259685 in 1% DMSO 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, or 30 μM, 100 μM, or 300 μM JNJ16259685 in 1% DMSO 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 5% CO_2. 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, 200 μl 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 (100 μM) or 4 independent experiments (DMSO, 30, 300 μM 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).

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.

Results

The continuous presence of glutamate promotes glioma cell line viability.

To evaluate the effects of glutamate on glioma cell line viability, exogenous glutamate was added to the growth media. Glioma cell lines (Hs683, A172, and U87) were incubated in standard media conditions (media and serum containing glutamate; termed: full media), media with dialyzed serum (termed: dialyzed media), and media with dialyzed serum supplemented with increasing concentrations of glutamate (Figure 1A). Following a five-day incubation period, cellular viability was measured using the MTT assay. As shown in Figure 1A, 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 mGluR1, SK2 (EC_{50}= 4.3 mM) and SK5 (EC_{50} 3.4 mM) (Gelb, et al., 2015a). 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% (± 4), 45% (± 7), and 48% (± 11) of full media controls, respectively (Figure 1D). However, when dialyzed media was supplemented with 20mM glutamate, viability returned to 79% (± 13), 93% (± 18), and 90% (± 7), of full media controls in Hs683, A172, and U87 cell lines, respectively (Figure 1D).

Recent reports indicate that the MTT assay may have off-target effects leading to inaccurate measurements of cellular viability under some treatment conditions (Jo, et al., 2015; Stepanenko and Dmitrenko, 2015). 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 comparable results when measuring the effect of glutamate supplementation on cellular viability (Supplemental Figure 3A and C). Likewise, when Hs683 viability was measured using the CyQUANT assay (after three days of treatment), the results were analogous to viability measured by the MTT assay (after five days of treatment) (Supplemental Figure 3B 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 (Ye and Sontheimer, 1999; Buckingham, et al., 2011; Takano, et al., 2001; Lyons, et al., 2007). We confirmed this finding and quantified the release of glutamate by cell lines used in this study (Figure 1B). Glutamate concentrations measured 5 days after plating in dialyzed media were 278 μM (± 22), 235 μM (± 27), and 246 μM (± 23), in Hs683, A172, and U87 cells, respectively, as measured by the Amplex Red assay. Concentrations within this range have been shown to promote survival in cells known to express high levels of mGluR1 protein, such as CHO cells heterologously expressing mGluR1 (EC_{50} = 153 μM) and cerebellar granule neurons (EC_{50}= 76 μM) (Emery, et al., 2010; Hathaway, et al., 2015). 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 data in Figure 1A.

To address this confounding factor, glutamate pyruvate transferase (GPT) was added to full media to enzymatically remove glutamate as it was released (Figures 1C and 1D). GPT removes glutamate by catalyzing the conversion of glutamate and pyruvate to alpha-

ketoglutarate and alanine; respectively. In the presence of GPT, Hs683 viability was dose dependently reduced (IC_{50} 28 μg/ml (CI 95% (4-181)) (Figure 1C). The dependence on glutamate, as revealed by GPT, was observed in all cell lines tested. Viability was reduced to 27% (± 5), 41% (± 4), and 34% (± 4) of full serum controls in Hs683, A172, and U87 cell lines, respectively (Figure 1D). As expected, in cells treated with high concentrations of GPT, viability was lower compared with cells cultured in dialyzed media only (Figure 1C). 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 glioma cell lines are dependent on glutamate for sustained viability.

mRNA encoding mGluR1 was confirmed in five human glioma cell lines by reverse transcription polymerase chain reaction (RT-PCR).

Using RT-PCR, we tested the expression of native mGluR1 mRNA in multiple glioma cell lines. Primers designed to detect mGluR1a and mGluR1b mRNA transcripts amplified 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 2). The expression level varied among the cell lines. For example, H4 cells produced an mGluR1a band just above the threshold of detection, while robust mGluR1a signals were observed in the other cell lines (Figure 2). However, the band intensity of the loading control, GAPDH, was consistent, indicating equal starting concentrations of cDNA. These results suggest that the mGluR1 gene, GRM1, is actively transcribed in human derived

glioma cells and that the receptor transcription level varies among the various glioma cell lines. It would have been ideal to also confirm the expression of mGluR1 protein. Unfortunately, we found that all currently available commercial mGluR1 antibodies lacked specificity for human mGluR1. Therefore, we were not able to successfully complete these experiments. Should an effective human mGluR1 antibody become available, it would be important to confirm protein expression.

β-arrestin dependent mGluR1 signaling may contribute to glioma cell line viability.

Studies conducted in our laboratory have suggested a positive impact of mGluR1 dependent signaling on cellular viability in several models including CHO cells heterologously expressing mGluR1, cerebellar granule neurons, and melanoma cell lines (Gelb, et al., 2015b; Emery, et al., 2010; Hathaway, et al., 2015). In addition, these studies have consistently concluded that ligand bias exists at mGluR1 and that: 1) unbiased ligands, such as glutamate and aspartate, can activate either the canonical $G\alpha_q$ pathway or a β-arrestin-dependent pathway that increases cellular viability and 2) biased ligands such as quisqualate and DHPG can only activate the $G\alpha_q$ pathway and do not have a positive impact on cellular viability (Gelb, et al., 2015b; Emery, et al., 2010; Emery, et al., 2012; Hathaway, et al., 2015). Therefore, we tested if mGluR1 signaling in glioma followed a similar pattern. Hs683 cells were cultured in full media (viability= 100%), dialyzed media (viability = 36% (± 2)), or dialyzed media in the presence of various mGluR1 agonists for five days and then subjected to the MTT assay (Figure 3A). As expected, 20 mM glutamate acted as a full agonist in dialyzed media with viability measured at 84% (± 5) of full serum controls. Aspartic acid was 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 EC_{50} values, respectively (DHPG EC_{50} = 10 μM and quisqualate EC_{50} = 1 μM) (Emery, et al., 2012). For example, 1 mM DHPG treated cells had a viability of 31% (± 2) and 300 μM quisqualate treated cells had a viability of 38% (± 6) compared to full serum controls (Figure 3A). Similar results were obtained using A172 and U87 cell lines (Supplemental Figure 4). Below, we present pharmacologic and genetic evidence indicating that mGluR1 specifically mediates glioma cell viability. In this context, these agonist viability results suggest that ligand bias may also occur at mGluR1 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 (Macia, et al., 2006). We found that dynasore dose-dependently blocked the positive effect of glutamate on glioma cell line viability in Hs683 cells with an IC_{50} of 31.8 μM (CI 95% (24.1-42.1)) (Figure 3B). In contrast, when increasing concentrations of dynasore were added to the cells grown in 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.

In the presence of glutamate, selective antagonists of mGluR1 negatively impact the viability of glioma cell lines in a dose-dependent manner.

After confirming active transcription of the mGluR1 gene in multiple glioma cell lines

and the dependence of glioma cell line viability on non-biased mGluR1 agonists such as glutamate and aspartate, we then examined the effect of mGluR1 antagonists in this model. Hs683 cells cultured in full media and treated with increasing concentrations of the non-competitive mGluR1 antagonist JNJ16259685 (JNJ), demonstrated reduced viability with an IC_{50} of 192 μM (CI 95% (138-266)) (Figure 4A). Similarly, when Hs683 cells were grown 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 4B). These IC_{50} values were higher than the widely reported IC_{50} for inhibition of $G\alpha_q$ signaling as measured by PI hydrolysis (0.5 nM) (Lavreysen, et al., 2004) but were similar to the reported IC_{50} for inhibition of cell viability in mGluR1 positive melanoma cell lines SK2 (109 μM) and SK5 (105 μM) (Gelb, et al., 2015a) and in cerebellar granule neurons (19.8 μM) (Hathaway, et al., 2015). 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 4C). Conversely, when increasing concentrations of JNJ (Figure 4B) or CPCCOEt (Figure 4C) were applied to the cells in dialyzed media without supplemental glutamate and with GPT added to enzymatically remove secreted glutamate, the data did not fit to a dose response curve. This suggests the antagonist mediated reduction in viability was not the result of a non-specific toxicity and could be attributed to antagonism of mGluR1 signaling.

Our findings were consistent in all glioma cell lines tested. JNJ and CPCCOEt responses were tested at two concentrations in the presence of 20 mM glutamate and dialyzed media (Figure 4D). In A172 cells, 100 μM and 300 μM JNJ 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 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 mGluR1 has not been shown to be expressed in astrocytes (Aronica, et al., 2003) or to be functionally relevant to their physiology, the other group I metabotropic receptor, mGluR5, which shares considerable sequence homology with mGluR1, is highly expressed in astrocytes (Loane, et al., 2012). mGluR5 has also been shown to be up-regulated in astrocytes in pathological conditions including epilepsy, multiple sclerosis, and amyotrophic lateral sclerosis (Loane, et al., 2012). 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, mGluR5 antagonist, was applied to Hs683, A172, and U87 cells in concentrations 10 and 1000-fold higher than the IC_{50} reported for MPEP at mGluR5 (36 nM) (Gasparini, et al., 1999). As shown in Supplemental Figure 5, 300 nM and 30 μM 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 the protective effect of glutamate is most likely selectively modulated by mGluR1.

mGluR1 shRNA reduces glioma cell line proliferation.

Having established that selective mGluR1 antagonists decrease glioma viability, we then utilized a gene silencing approach to confirm our pharmacological data. Four plasmids, each encoding shRNA targeting mGluR1 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 5A). As expected, mGluR1a and mGluR1b amplicons were detected in Hs683 glioma cells transfected with a scrambled shRNA control plasmid. In contrast, mGluR1a and mGluR1b were effectively silenced in Hs683 glioma cells transfected with 4 different mGluR1 targeted shRNA plasmids.

Following confirmation of effective mGluR1 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 mGluR1 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 (Supplemental Figure 3B).

Measuring the proliferative activity over time in 96 well plates, we found Hs683 cells treated with mGluR1 shRNA had significantly less nucleic acid content per well compared with those treated with a scrambled shRNA control or with transfection agent alone at 72 hours (Figure 5B). Also, no difference was noted between Hs683 cells exposed to transfection agent only and those transfected with the scrambled shRNA control plasmid at 72 hours, validating the specificity of mGluR1 shRNA effect. These results demonstrate that mGluR1 knock-down specifically reduced the proliferation of glioma cells over time and strongly support the hypothesis that mGluR1 plays a significant role in the proliferative activity and viability of

glioma cells *in vitro*.

The mGluR1 antagonist, JNJ16259685, 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 (Freedman and Shin, 1974; Mori, et al., 2009). 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 35 mm well following a three-week incubation in soft agar; while "extreme" anchorage-dependent growth has been defined as less than 20 colonies (Mori, et al., 2009). 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 29 μm^2 (± 5) (Figure 6A and B). However, when the non-competitive mGluR1 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 (20 μm^2 (± 2)) could be seen with 30 μM 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 12 μm^2 (± 2) (Figure 6 A and B). Furthermore, an average of 4 (± 1) colonies were detected when Hs683 was cultured in the presence of 300 μM JNJ, indicating a conversion to an "extreme" anchorage-dependent growth phenotype. These results suggest non-competitive antagonism of mGluR1 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.

Discussion

A variety of cancerous cells release glutamate including melanoma (Namkoong, et al., 2007), breast carcinoma (Ungard, et al., 2014; Seidlitz, et al., 2009), prostate carcinoma (Seidlitz, et al., 2009) and glioma (Ye and Sontheimer, 1999; Takano, et al., 2001; Lyons, et al., 2007). 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 and glutamate dose dependently promoted glioma cell viability (Figure 1).

Using RT-PCR, mGluR1 splice variant transcripts, 1a and 1b, were detected in all glioma cell lines tested (Figure 2), validating previous reports of mGluR1 mRNA expression in glioma (Stepulak, et al., 2009; Brocke, et al., 2010; Parsons, et al., 2008) and consistent with a study of mGluR1 mRNA expression in melanoma (DiRaddo, et al., 2013). Four additional splice variants for mGluR1 have been described (DiRaddo, et al., 2013). Assuming one, or more, of other isoforms of mGluR1 are expressed, it would be important to understand which splice variants increased glioma proliferation. It will also be important to obtain a mGluR1 antibody with adequate specificity to confirm mGluR1 protein expression.

It is well established that glutamate activation of mGluR1 induces a $G\alpha_q$-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 ionotropic receptor currents (Ferraguti, et al., 2008), and transient ERK phosphorylation (Emery, et al., 2010; Emery, et al., 2012). Based upon this characterization of mGluR1 signaling, receptor activity is often stimulated with agonists known

to stimulate $G\alpha_q$ signaling and measured using $G\alpha_q$ signaling pathway outputs such as intracellular calcium concentration and PI hydrolysis (Gelb, et al., 2015b). However, our laboratory has reported a mechanism by which glutamate-stimulated mGluR1 mediates cell survival that would not be captured by $G\alpha_q$ signaling outputs (Gelb, et al., 2015b). This mechanism involves a $G\alpha_q$ independent, β-arrestin dependent, signaling cascade that induces sustained ERK phosphorylation and improved cellular viability (Emery, et al., 2010). Furthermore, our laboratory has observed evidence of ligand bias at mGluR1 expressed in CHO cells, melanoma cells, and in cerebellar granule neurons (CGN) (Gelb, et al., 2015b; Emery, et al., 2012; Hathaway, et al., 2015). In these models, unbiased ligands, such as glutamate and aspartate, activate either the $G\alpha_q$ pathway or the β-arrestin-dependent pathway and biased ligands such as quisqualate and DHPG only activate the $G\alpha_q$ pathway (Gelb, et al., 2015b; Emery, et al., 2012; Hathaway, et al., 2015). We observed a similar pattern in glioma cell lines (Figure 3A and Supplemental Figure 4) consistent with the hypothesis that mGluR1 demonstrates ligand bias across several cellular models.

In this study, we confirmed that glioma cells release high concentrations of glutamate into the surrounding culture media (Figure 1B). This prolonged cellular exposure to glutamate is likely to influence mGluR1 signaling by stimulating homologous receptor desensitization, a process that includes β-arrestin and dynamin dependent receptor internalization (Ferraguti, et al., 2008; Dhami and Ferguson, 2006). Theoretically, internalized mGluR1 would not be available at the cell surface to signal through the $G\alpha_q$ signaling cascade but could signal through the β-arrestin dependent pathway, ultimately promoting glioma cell viability. Several studies of mGluR1 desensitization and internalization in heterologous models support this hypothesis

(Ferraguti, et al., 2008; Dhami and Ferguson, 2006; Sallese, et al., 2000; Mundell, et al., 2001; Doherty, et al., 1999).

To determine if glutamate-stimulated viability was dependent on receptor internalization, we used the dynamin inhibitor, dynasore. Dynamin is required for endocytosis of clathrin-coated pits containing mGluR1: β-arrestin complexes; an event preceding β-arrestin dependent signaling (Macia, et al., 2006; Ferraguti, et al., 2008; Dhami and Ferguson, 2006). We found that dynasore dose dependently blocked the positive effect of glutamate on glioma cell line viability (Figure 3B). While off target effects of dynasore have been reported, including disruptive effects in the regulation of intracellular cholesterol and lipid raft structure (Preta, et al., 2015), we found no change in viability when increasing concentrations of dynasore were added to dialyzed media (with GPT added to remove secreted glutamate). This suggests that the observed effect was specifically associated with blockade of glutamate-stimulated effects. Based on these results, it is likely that receptor internalization is required for glutamate to promote glioma viability; further supporting our hypothesis that this effect may be β-arrestin dependent.

In this study, the selective mGluR1 non-competitive antagonists JNJ16259685 (JNJ) and CPCCOEt dose dependently reduced glutamate-stimulated glioma cell viability (Figure 4) with values consistent with the reported IC_{50} for inhibition of viability in mGluR1 positive melanoma cell lines (Gelb, et al., 2015a) and in CGN (Hathaway, et al., 2015). The IC_{50} values for viability are significantly higher than those reported for inhibition of Gαq mGluR1 signaling (1.2-19 nM for JNJ (Knöpfel, 2007) and 6.5 μM for CPCCOEt (Litschig, et al., 1999)). It has been suggested that the difference in IC_{50} values for antagonists inhibiting mGluR1 signaling via Gαq versus β-arrestin may reflect "biased antagonism;" where the noncompetitive antagonists have an increased potency for receptor conformations that inhibit Gαq signaling compared with receptor

conformations that inhibit glutamate-dependent viability effects (Hathaway, et al., 2015). Another possibility exists in glioma, where sustained elevations in local glutamate concentration at mGluR1 would be expected to induce β-arrestin dependent receptor internalization. In this context, it is likely that higher concentrations of antagonist are required to overcome the cell membrane barriers to reach internalized mGluR1.

There is substantial evidence that both ionotropic and other metabotropic glutamate receptors promote glioma proliferation, migration, and survival (Takano, et al., 2001; Lyons, et al., 2007; Ishiuchi, et al., 2007; Ishiuchi, et al., 2002; Pereira, et al., 2017; Ciceroni, et al., 2013; Nicoletti, et al., 2007; Arcella, et al., 2005; Zhou, et al., 2014; D'onofrio, et al., 2003). Therefore, it is possible that the higher doses of noncompetitive antagonists required to affect viability 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 5B) compared with scrambled shRNA controls. This validates our pharmacological data and suggests that mGluR1 is specifically involved in glioma proliferation.

Anchorage independent growth is a widely used, highly reliable test of malignant transformation (Borowicz, et al., 2014; Mori, et al., 2009). The assay measures cellular proliferation in the absence of extracellular matrix binding, predicting an *in vivo* capability to form metastatic tumors (Freedman and Shin, 1974; Mori, et al., 2009). When we cultured Hs683 cells in soft agar, the cells exhibited significant anchorage-independent growth (Figure 6). However, as increasing concentrations of JNJ were added to the soft agar, both colony size and number were reduced, indicating a conversion to an anchorage-dependent phenotype. A trend towards decreased colony size was observed at 30 μM JNJ, and a significant difference in both

colony size and number were measured at 100 μM, concentrations that had minimal effects on cell viability (compare Figures 4 and 6). Therefore, the effects observed at 100 μM JNJ in soft agar are likely to reflect an inhibition of anchorage dependent growth, rather than a reduction in proliferation or viability in general. These results suggest that noncompetitive antagonism of mGluR1 may decrease the tumorigenic and metastatic potential of glioma cells *in vivo*.

A common finding in cancer genetics is the "selective re-expression" (Coggin and Anderson, 1974) of genes that regulate early growth and development (Ligon, et al., 2017). mGluR1 has been associated with neurogenesis, including the enhancement of neural progenitor cell proliferation (Friedman, et al., 2016; Baskys, et al., 2005; Castiglione, et al., 2008). Furthermore, mGluR1 appears to regulate oxidative stress in immature oligodendrocytes, with expression levels dropping dramatically with development (Loane, et al., 2012; Deng, et al., 2004). In the present study, we detected mGluR1 mRNA in the Hs683 glioma cell line, an oligodendroglioma with a GBM phenotype (Le Mercier, et al., 2009; Bruyère, et al., 2011). We also detected mGluR1 mRNA expression in cell lines classified as high-grade astrocytoma or GBM (Figure 2; U87, A172, and U118). The consistent expression of mGluR1 mRNA in high grade astrocytoma (Stepulak, et al., 2009; Brocke, et al., 2010; Parsons, et al., 2008) is somewhat surprising because mGluR1 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 (D'Antoni, et al., 2008; Agrawal, et al., 1998; Aronica, et al., 2001). These finding parallel what has been seen in melanoma, where mGluR1 is not detected in melanocytes but is detected in the malignant phenotype, melanoma (Shin, et al., 2008; Wangari-Talbot, et al., 2012). Furthermore, there is substantial evidence from our laboratory (Gelb, et al., 2014: Gelb, et al., 2015a; Gelb, et al., 2015b) and others to suggest that mGluR1 acts as a proto-oncogene when aberrantly expressed in

melanoma (Marín, et al., 2006; Namkoong, et al., 2007; Shin, et al., 2008) and breast cancer (Speyer, et al., 2008; Banda, et al., 2014). Among the most convincing evidence, is the report that mGluR1 cDNA transfection into melanocytes transforms the cells to a melanoma phenotype which can be subsequently reversed with knock-down of the receptor (Shin, et al., 2008). In the present study, we exclusively focused on glioma cell lines to provide evidence that mGluR1 may play a similar role in yet another form of malignancy. We demonstrate that glioma cell viability is dependent on glutamate (Figure 1), and that blockade of mGluR1 signaling with selective non-competitive antagonists (Figure 4), and with genetic silencing (Figure 5) significantly reduces glutamate-stimulated glioma cell viability and proliferation. In addition, we demonstrate that the non-competitive selective mGluR1 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 6). Taken together, these results strongly suggest that mGluR1 may act as a proto-oncogene in glioma by promoting dysregulated proliferation and survival of glioma cells in a manner similar to the role of mGluR1 in melanoma. This effect may be the result of "selective re-expression" of GRM1, the mGluR1 gene, which in normal developmental physiology, drives the proliferation of neural progenitor cells (Friedman, et al., 2016; Baskys, et al., 2005; Castiglione, et al., 2008) and promotes the survival of immature oligodendrocytes (Deng, et al., 2004). Thus, inhibition of mGluR1 signaling may offer an alternative strategy for treating glioma. Future testing in translational models will be important to determine if mGluR1 signaling can provide an opportunity to diversify GBM treatment in an effort to combat treatment resistance and improve the prognosis of this devastating disease. In addition, based on these findings in glioma, and those in melanoma and breast cancer, a

comprehensive screen of mGluR1 involvement across other malignancies is warranted.

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Authorship Contributions

Participated in research design: Bowman Dalley, Wroblewska, Wolfe, and Wroblewski.

Conducted experiments: Bowman Dalley and Wroblewska.

Performed data analysis: Bowman Dalley, Wroblewska, Wolfe, and Wroblewski.

Wrote or contributed to the writing of this manuscript: Bowman Dalley, Wroblewska, and Wolfe.

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Footnotes

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‡ Jarda T. Wroblewki, PhD, passed away before submission of this paper. He was actively involved in experimental design and data analysis prior to his death.

Figure Legends

Figure 1: Glutamate promotes the viability of glioma cell lines. (A) Hs683 glioma cell viability in the presence of glutamate as compared to dialyzed media. The dashed line represents Hs683 cellular viability in dialyzed, glutamate free media (A and C). (B) Measurement of glutamate levels in lysates of Hs683, A172, and U87 cells after 1, 3, and 5 days of culture was performed using an Amplex Red assay. (C) Hs683 cell viability when glutamate is continuously removed from the media by increasing doses of glutamate-pyruvate transaminase (GPT) in the presence of 10 mM pyruvate. (D) Comparative viability of Hs683, A172, and U87 cells grown in full media, dialyzed media, dialyzed media supplemented with 20mM glutamate (glu) and full media supplemented by GPT. In viability assays (A, C, and D), 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 7. All data in A, C, and D were normalized to viability in full media without GPT. Data points in all panels represent the mean of at least three independent experiments (n) (± SEM) measured in triplicate. For (A) and (C), the line represents data fit with GraphPad Prism 7.0 using the non-linear regression, variable slope, four-parameter equation. In (B), a two-way ANOVA with Tukey's Multiple Comparison Test was used (**$p<0.01$ comparing day 5 with day 1). In (D), the data were normalized to the mean of the full media group within each experiment and a one sample t-test was used to compare cells cultured in full media with those cultured in dialyzed media (*$p<0.05$). Then a one-way ANOVA with Dunnett's Multiple Comparisons Test was used to compare the dialyzed group to the treatment groups (*$p<0.05$).

Figure 2: Human glioma cell lines express messenger RNA for the mGluR1a & mGluR1b receptor. (A) Representative 2% agarose gel containing RT-PCR products from Hs683, H4,

U87, A172 and U118 cell lines as compared to CHO cells stably transfected with mGluR1 cDNA. (B) PCR product intensity of this representative experiment was quantified using Image J and normalized to a matched human GAPDH control. GAPDH and mGluR1 are products of separate reactions that included equivalent volumes of the same aliquot of cDNA. Photographs were cropped to promote clarity.

Figure 3: Glioma cell viability is promoted by unbiased mGlu1 receptor agonists and blocked by the inhibitor of receptor internalization (dynasore). (A) Comparative viability of Hs683 cells grown in the presence of either full media, dialyzed media, or dialyzed media with glutamate (Glu), aspartate (Asp), DHPG or quisqualate (Quis). (B) Hs683 cell viability when increasing concentrations of dynasore were added to dialyzed media with either supplemental glutamate or GPT (added to enzymatically remove glioma secreted glutamate). Cellular viability was measured using the MTT assay and data was normalized to viability in full serum. Each data point represents the mean (± SEM) of at least 3 independent experiments (n) measured using the MTT assay in triplicate or more. In (A), one-way ANOVA with Dunnett's Multiple Comparisons Test was utilized to measure the differences between groups (***$p<0.001$, comparing to viability in dialyzed media). In (B) non-linear regression was used to fit the data using a variable slope, four parameter-equation.

Figure 4: Selective non-competitive antagonists of the mGluR1 decrease the viability of Hs683 cells in a dose dependent manner. Hs683 cells cultured in full media (A) or dialyzed media supplemented with 20 mM glutamate (glu) (B) and treated with increasing concentrations of the non-competitive mGluR1 antagonists JNJ16259685 (JNJ) or CPCCOEt (CPC). (C) In the

experiments utilizing dialyzed media, a control condition was included where GPT was added to enzymatically remove glioma secreted glutamate. (D) Comparative viability of Hs683, A172, and U87 cells grown in dialyzed media or dialyzed media supplemented with 20mM glutamate (glu) and different concentrations of the antagonists. Cellular viability was measured using the MTT assay and data was normalized to viability in full serum. 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 (A, B, and C) and one-way ANOVA with Dunnett's Multiple Comparisons Test was utilized to measure the differences between groups (D) (*$p<0.05$, **$p<0.01$, ***$p<0.001$ comparing to viability in dialyzed media + 20 mM glutamate).

Figure 5: GRM1 gene silencing with shRNA significantly reduced glioma cell proliferation. (A) 2% agarose gel containing PCR products from Hs683 glioma cells transfected with a scrambled shRNA control plasmid or 4 different mGluR1 targeted shRNA plasmids. GAPDH and mGluR1 products were generated in separate reactions that included equivalent volumes of the same aliquot of cDNA. Photographs were cropped to promote clarity. (B) Hs683 cells were transfected with the shRNA plasmids, scrambled shRNA control or with transfection agent alone (wild type). Nucleic acid content was measured using the CyQUANT Cell Proliferation Kit 24, 48 and 72 hours after the transfected cells were plated on 96 well plates. 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. At 72 hours, a one-way ANOVA with Tukey's Multiple Comparisons Test was used to measure the differences between groups (**$p<0.01$, comparing wild type and control

scrambled shRNA to mGluR1 shRNA).

Figure 6: The non-competitive mGluR1 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 (100 μM) or 4 independent experiments (control, 30, 300 μM 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. One-way ANOVA with Dunnett's Multiple Comparisons Test was utilized to measure the differences between groups (**$p<0.01$, ***$p<0.001$ comparing relative colony number or average colony size to the mean of the control group).

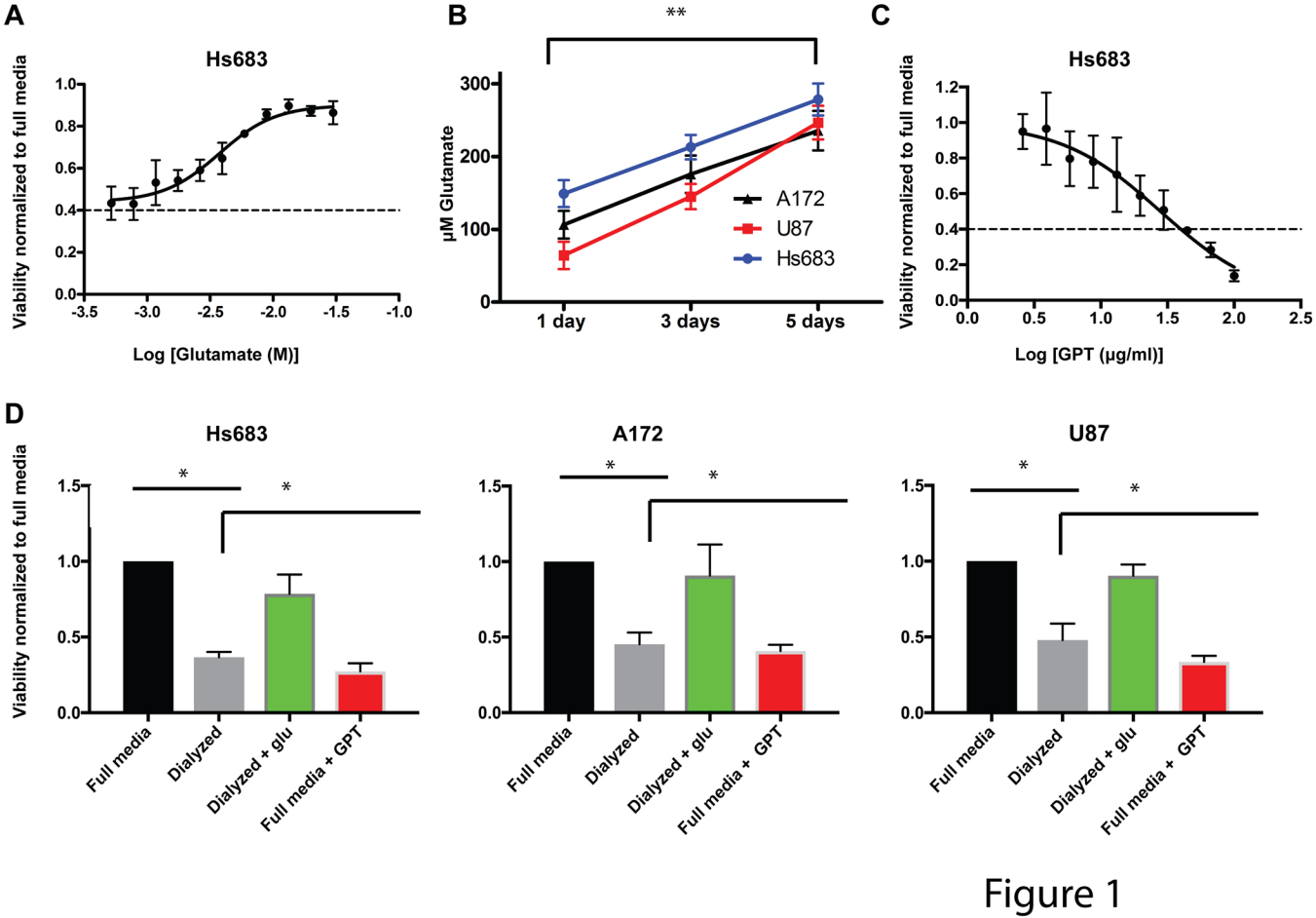
A
Hs683
Viability normalized to full media
1.0
0.8
0.6
0.4
0.2
0.0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
Log [Glutamate (M)]
B
**
μM Glutamate
300
200
100
0
1 day
3 days
5 days
A172
U87
Hs683
C
Hs683
Viability normalized to full media
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
Log [GPT (μg/ml)]
D
Hs683
A172
U87
Viability normalized to full media
1.5
1.0
0.5
0.0
*
*
Full media
Dialyzed
Dialyzed + glu
Full media + GPT

Figure 1

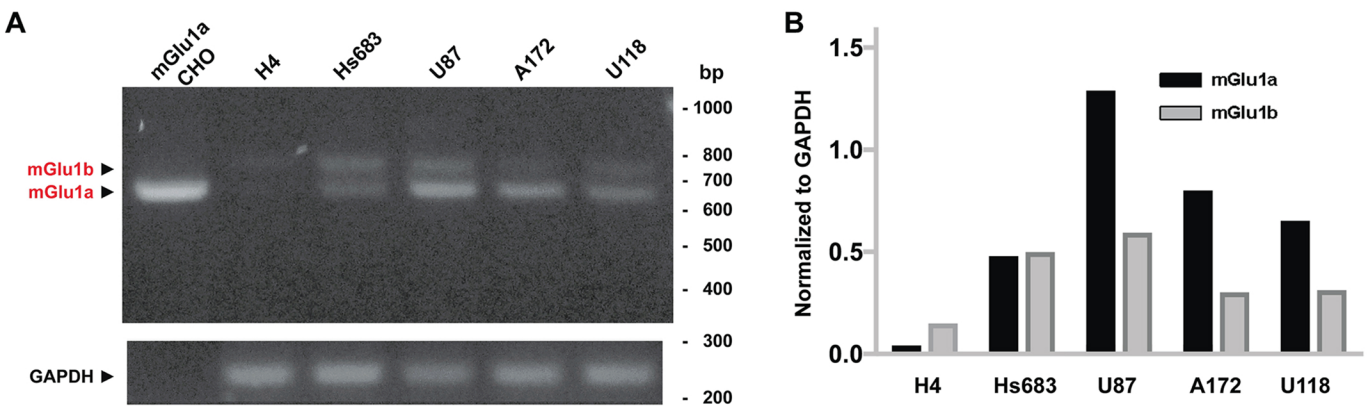
A
mGlu1a CHO
H4
Hs683
U87
A172
U118
bp
1000
800
700
600
500
400
300
200
mGlu1b
mGlu1a
GAPDH
B
Normalized to GAPDH
1.5
1.0
0.5
0.0
mGlu1a
mGlu1b
H4
Hs683
U87
A172
U118

Figure 2

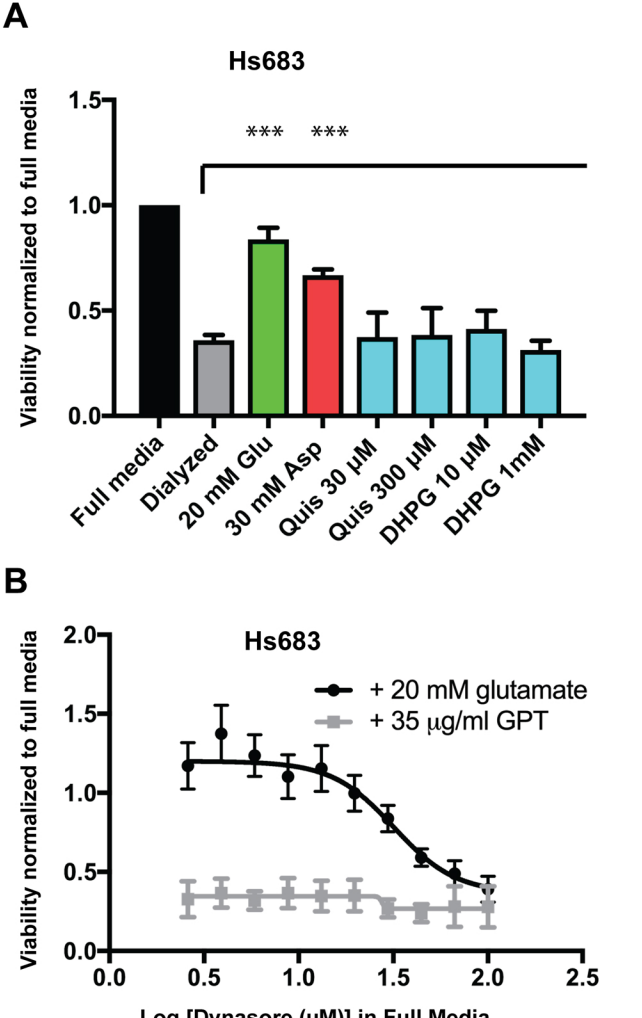
A
Hs683
Viability normalized to full media
1.5
1.0
0.5
0.0
*** ***
Full media
Dialyzed
20 mM Glu
30 mM Asp
Quis 30 µM
Quis 300 µM
DHPG 10 µM
DHPG 1mM
B
Hs683
Viability normalized to full media
2.0
1.5
1.0
0.5
0.0
+ 20 mM glutamate
+ 35 µg/ml GPT
0.0
0.5
1.0
1.5
2.0
2.5
Log [Dynasore (µM)] in Full Media

Figure 3

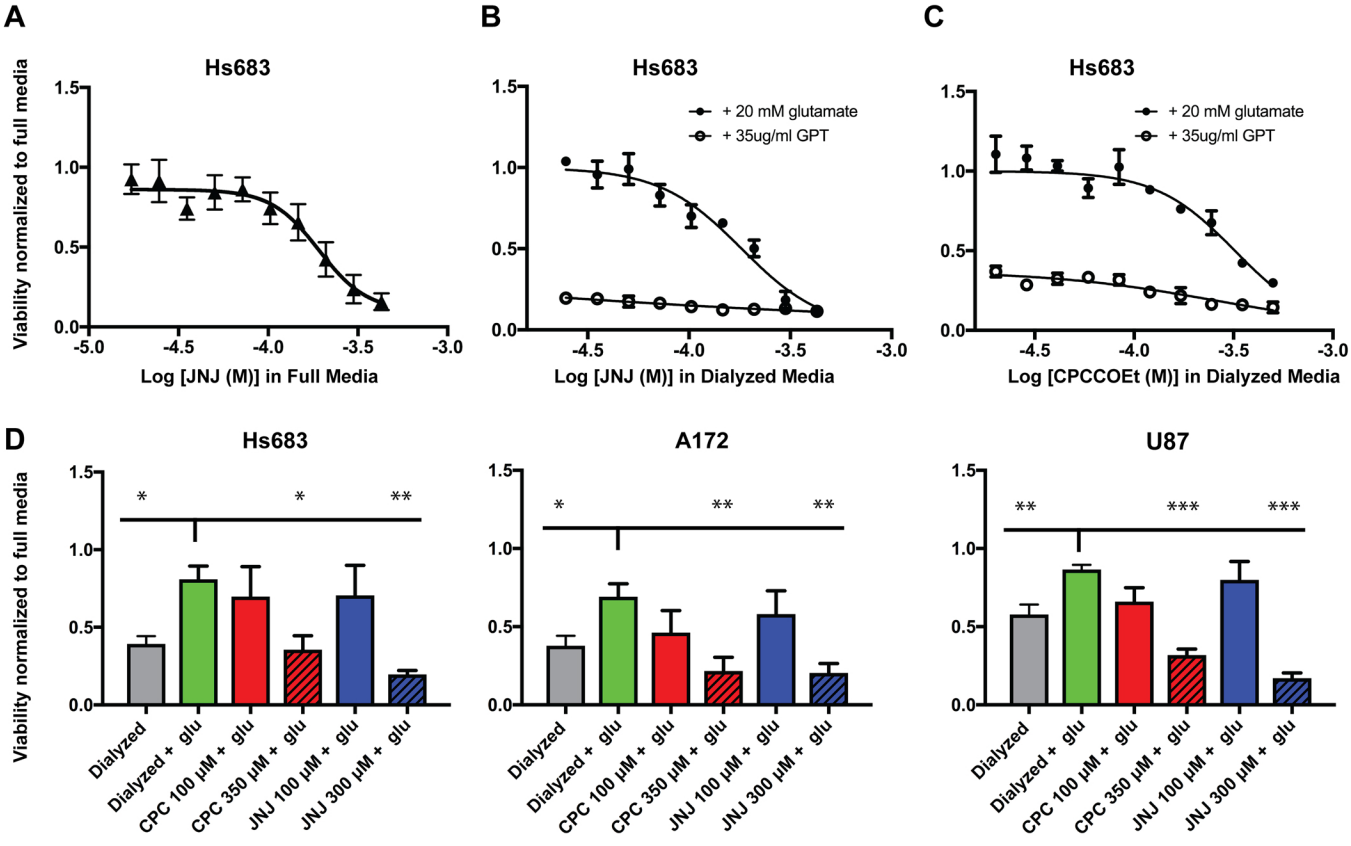
A
Hs683
Viability normalized to full media
Log [JNJ (M)] in Full Media
B
Hs683
+ 20 mM glutamate
+ 35ug/ml GPT
Log [JNJ (M)] in Dialyzed Media
C
Hs683
+ 20 mM glutamate
+ 35ug/ml GPT
Log [CPCCOEt (M)] in Dialyzed Media
D
Hs683
A172
U87
Viability normalized to full media
Dialyzed
Dialyzed + glu
CPC 100 µM + glu
CPC 350 µM + glu
JNJ 100 µM + glu
JNJ 300 µM + glu

Figure 4

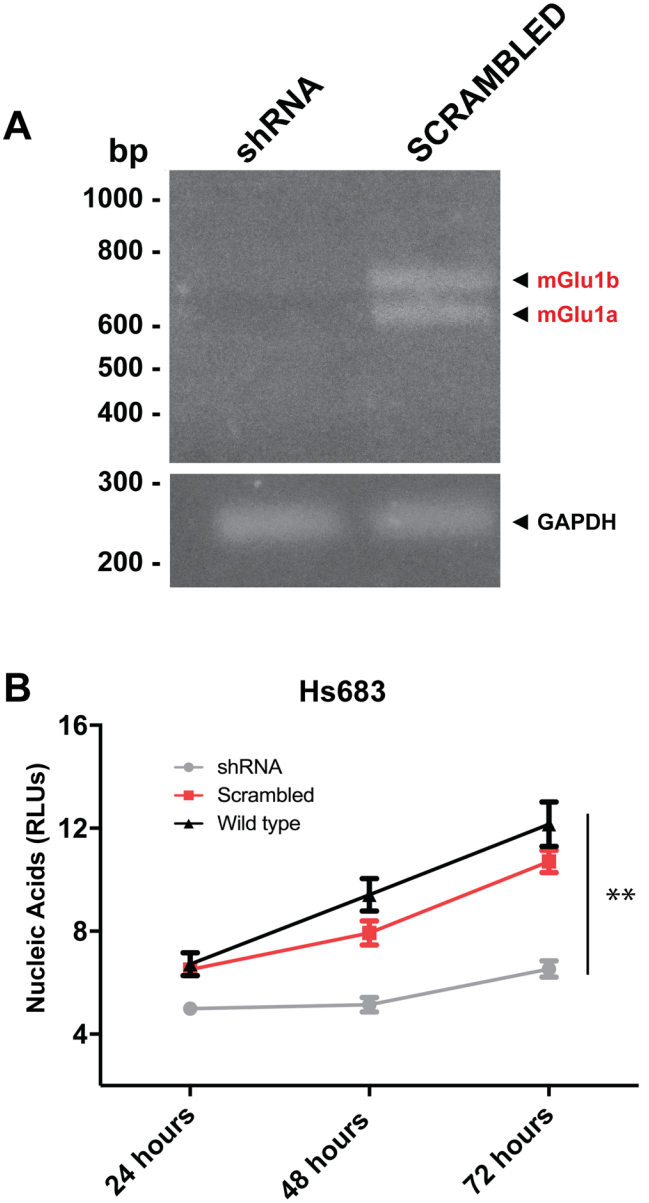
A
bp
shRNA
SCRAMBLED
1000
800
600
500
400
300
200
mGlu1b
mGlu1a
GAPDH
B
Hs683
Nucleic Acids (RLUs)
16
12
8
4
shRNA
Scrambled
Wild type
**
24 hours
48 hours
72 hours

Figure 5

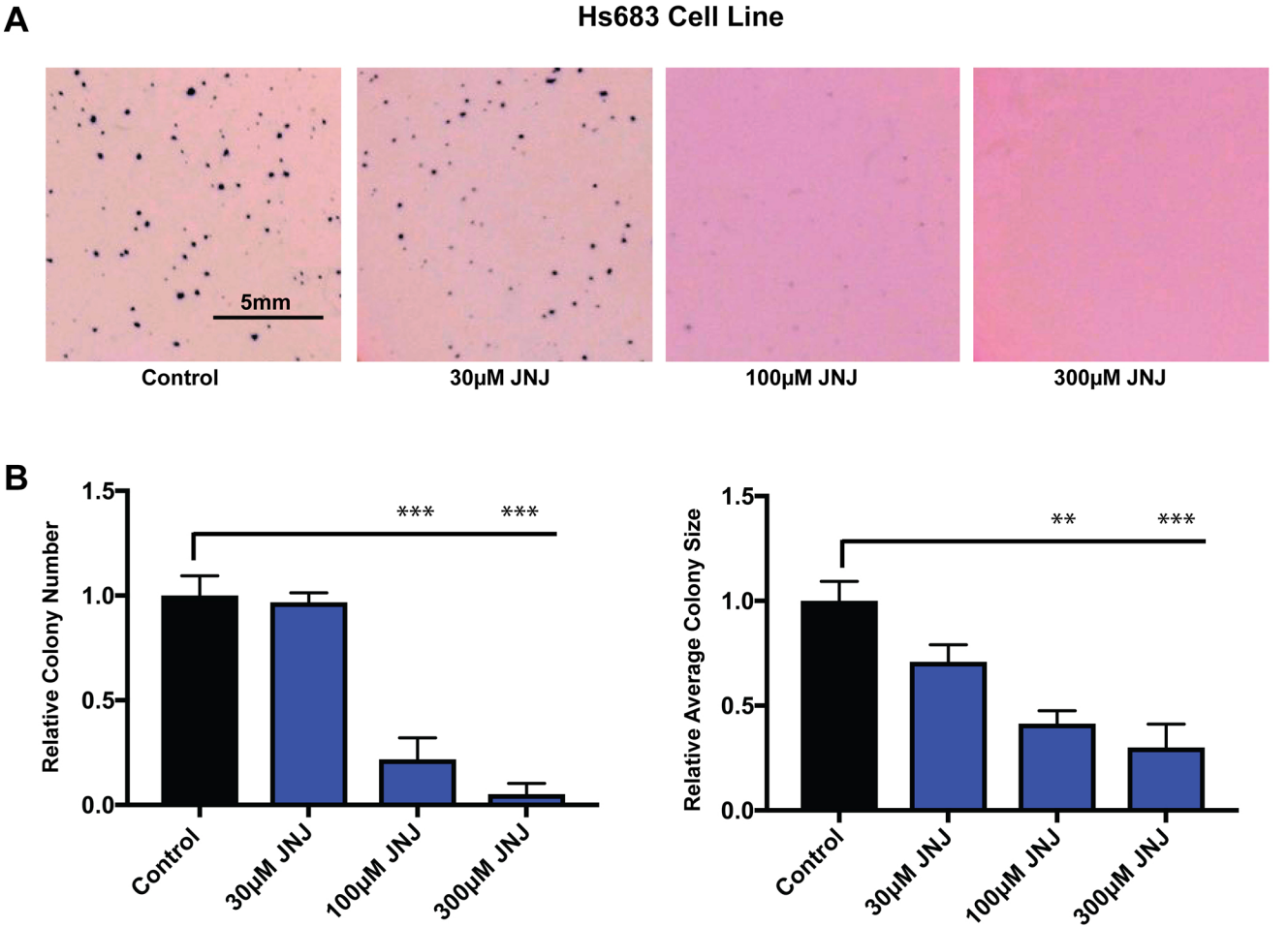
A
Hs683 Cell Line
5mm
Control
30μM JNJ
100μM JNJ
300μM JNJ
B
Relative Colony Number
1.5
1.0
0.5
0.0
***
***
Control
30μM JNJ
100μM JNJ
300μM JNJ
Relative Average Colony Size
1.5
1.0
0.5
0.0
**
***
Control
30μM JNJ
100μM JNJ
300μM JNJ

Figure 6