



HHS Public Access

Author manuscript

ACS Chem Neurosci. Author manuscript; available in PMC 2020 March 27.

Published in final edited form as:

ACS Chem Neurosci. 2019 August 21; 10(8): 3437–3453. doi:10.1021/acscchemneuro.9b00061.

Novel positive allosteric modulators of glutamate transport have neuroprotective properties in an *in vitro* excitotoxic model

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Abstract

Dysfunction of excitatory amino acid transporters (EAATs) has been implicated in the pathogenesis of various neurological disorders, such as stroke, brain trauma, epilepsy, and several neurodegenerative disorders. EAAT2 is the main transporter subtype responsible for glutamate clearance in the brain, and plays a key role in regulating neurotransmission and preventing excitotoxicity. Therefore, compounds that increase the activity of EAAT2 have therapeutic potential for neuroprotection. In previous studies, we used virtual screening approaches to identify novel positive allosteric modulators (PAMs) of EAAT2. These compounds were shown to selectively increase the activity of EAAT2 and increase V_{max} of transport, without changing substrate affinity. In this work, our major effort was to investigate whether increasing the activity of EAAT2 by allosteric modulation would translate to neuroprotection in *in vitro* primary culture models of excitotoxicity. To investigate potential neuroprotective effects of one EAAT2 PAM, GT949, we subjected cultures to acute and prolonged excitotoxic insults by exogenous application of glutamate, or oxidative stress by application of hydrogen peroxide. GT949 administration did not result in neuroprotection in the oxidative stress model, likely due to damage of the glutamate transporters. However, GT949 displayed neuroprotective properties after acute and prolonged glutamate-mediated excitotoxicity. We propose that this compound prevents excess glutamate signaling by increasing the rate of glutamate clearance by EAAT2, thereby preventing excitotoxic damage and cell death. This novel class of compounds is therefore an innovative approach for neuroprotection with potential for translation in *in vivo* animal models of excitotoxicity.

Keywords

EAAT2; glutamate transporter; glutamate uptake; transport enhancement; EAAT2 activators; glutamate excitotoxicity

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Author contributions

R.M.F., R.W., O.M., J.S. and A.C.K.F. designed the research. R.M.F., R.W. and J.E.G. conducted the experiments. R.M.F., R.W., J.E.G. and A.C.K.F. analyzed the data. R.M.F., R.W., O.M., J.S. and A.C.K.F. wrote the manuscript.

Conflict of interests

Compounds GT949 and GT996 are protected under a patent application submitted by Drexel University to USPTO (WO application WO2018132829A1) and includes named authors J.M.S. and A.C.K.F.

INTRODUCTION

Glutamate is the predominant excitatory amino acid neurotransmitter in the mammalian central nervous system (CNS), and is essential for normal brain functions including cognition, memory, learning, developmental plasticity, and long-term potentiation¹⁻³. Glutamate neurotransmission is terminated by presynaptic and astrocytic excitatory amino acid transporters (EAATs)⁴⁻⁶, which remove glutamate from the synaptic cleft. This process involves co-transport of glutamate and three sodium ions, followed by counter transport of one potassium⁷⁻⁹. There are five structurally distinct subtypes of Na⁺-dependent EAATs, including (rat/human homolog): GLAST/EAAT1/, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5, which allows for precise spatial and temporal regulation of glutamate neurotransmission⁴. The subtype GLT-1/EAAT2 plays a central role in maintenance of extracellular glutamate homeostasis and prevention of excitotoxicity¹⁰⁻¹⁴. EAAT2 accounts for approximately 95% of the total glutamate transport activity and 1% of total brain protein in the CNS¹⁵⁻¹⁹. This transporter is primarily expressed in astrocytes and select neurons and oligodendrocytes of the brain and spinal cord^{14, 20}.

Glutamate excitotoxicity is caused by sustained elevation of extracellular glutamate levels and activation of postsynaptic glutamate receptors, resulting in excessive Ca²⁺ influx²¹ and activation of a cascade of phospholipases, endonucleases, and proteases that can lead to apoptotic and necrotic cell death²²⁻²⁴. Sustained elevation of glutamate levels may occur due to failure of astrocytes or other cells to take up excess synaptic or ambient glutamate through EAATs. Glutamate excitotoxicity plays a key role in secondary damage following acute pathologies, including traumatic brain injury (TBI)²⁵⁻³¹, hyper-excitability and seizures^{32, 33}, stroke^{11, 34-37}, epilepsy^{33, 38, 39}, and cerebral and retinal ischemia^{16, 26, 27, 40-44}. Excitotoxicity also produces secondary damage in chronic pathologies, including Amyotrophic lateral sclerosis (ALS)^{45, 46}, Alzheimer's disease⁴⁷, Huntington's disease^{48, 49}, neuropathic pain⁵⁰ and HIV-associated neurocognitive disorders (HAND)⁵¹⁻⁵³.

Approaches that aim to mitigate excitotoxicity, such as glutamate receptor antagonists^{28, 54-61} and inhibition of calcium influx^{62, 63} alleviate cellular damage and neuronal deficits after brain trauma, but their use in the clinic has been severely limited due to serious side effects^{58, 64}. On the other hand, approaches that focus on removing excess glutamate through the stimulation of glutamate transporters may offer a safe and effective treatment for excitotoxicity. In this regard, approaches that increase EAAT2 protein expression^{13, 65} are currently being pursued. Several transcriptional or translational upregulators of EAAT2, such as GPI-1046⁶⁶, ceftriaxone^{37, 67-70}, harmine^{71, 72} and pyridazine derivatives^{73, 74} were shown to be neuroprotective through augmentation of glutamate transport. Although EAAT2 upregulation is a promising approach to treat excitotoxicity, it is most relevant for chronic conditions or prophylactic use⁷⁵.

Other approaches that relieve excitotoxicity by directly or indirectly increasing EAAT2 activity are also being pursued. Several compounds acutely stimulate glutamate uptake by indirect modulation of transporter activity, such as MS-153⁷⁶, riluzole^{77, 78}, guanosine^{79, 80},

and nicergoline⁸¹ resulting in neuroprotective outcomes. However, clinical use of these compounds is limited due to poor target specificity and side effects, highlighting the need for novel target-specific treatments. Previous studies from our group have identified a natural compound isolated from *Parawixia bistriata* spider venom that enhances glutamate uptake through EAAT2, and has neuroprotective properties^{82, 83}. Using this compound, we identified the molecular determinants of EAAT2 transport enhancement in a mutagenesis guided study⁸⁴. This unique structural information was then used in a virtual screening approach to identify molecules that interact with EAAT2 allosteric sites, using a structural model based on the 3D crystal structure of the bacterial EAAT2 homolog GltPh^{85–88}. These compounds were then characterized in glutamate uptake assays in transfected cells and in glial cultures, as allosteric modulators (AM) of EAAT2⁸⁹.

In this study, we evaluated potential neuroprotective properties of one of these EAAT2 positive allosteric modulators (PAM), GT949⁸⁹, in primary neuronal culture models of excitotoxicity. Our hypothesis is that positive allosteric modulation of EAAT2 activity will rapidly remove excessive glutamate from the synapse and significantly improve neuronal survival and disease outcome in excitotoxic conditions. This novel approach may result in a new generation of safe and effective therapies for CNS disorders with comorbid excitotoxicity.

RESULTS AND DISCUSSION

In this study, we evaluated GT949, a PAM of glutamate transporter EAAT2, for neuroprotective effects after acute or prolonged insults with application of L-glutamate or H₂O₂. Two primary culture approaches were used: a bilaminar neuron-glia co-culture and a mixed neuron-glia culture. As positive control for neuroprotection, we used AP-5 (DL-2-Amino-5-phosphonopentanoic acid), an NMDA receptor blocker. As negative control, we used GT996, an analog of GT949 that does not increase glutamate uptake⁸⁹. We exposed these cultures to glutamate or H₂O₂, and examined the neuroprotective potential of GT949 by immunocytochemical analysis, comparing MAP-2 expression⁹⁰ among different experimental groups.

Effects of GT949 in primary cultures subjected to acute glutamate-mediated excitotoxicity and oxidative stress insults

We began our analysis examining the effects of GT949 in a glutamate-mediated excitotoxicity model using a bilaminar co-culture system, where primary cortical neurons plated on coverslips were cultured in the presence of a glial feeder layer. This allowed us to separate the neurons and glia for treatments and downstream analysis as needed.

In the neuronal coverslips, ~97% of counted cells were MAP-2 positive, indicating that the vast majority of cells are neurons, and ~2–3% of cells were GFAP positive, indicating minimal astroglia contamination (< 3%, supplemental figure 1), which is in agreement with other studies using the same system⁹¹. The glial feeder is comprised of 100% GFAP positive cells, also in accordance to previous literature^{92, 93}.

To rule out any toxicity by compound GT949 in astrocytes, we incubated pure glia cultures with increasing concentrations of GT949 (up to 1 μM). Supplemental figure 2A shows images of pure glia cultures incubated with vehicle (control) and 1 μM GT949 for 24 hours, indicating preservation of glial morphology. The quantification of GFAP + cells in figure 2B indicates no toxicity of the compound, as there no significant changes between the number of cells between control (vehicle), 100 nM and 1 μM GT949.

Neuronal survival rates after acute glutamate insult and GT949 treatment in the presence of the glia—Neurons and glia were co-cultured for 14 DIV before treatments. At 14 DIV, there is substantial literature showing that glutamate receptors (NMDA and AMPA) are well expressed^{91, 94–96}. Supplemental figure 1 also demonstrates the differential expression of glutamate transporters at 14 DIV, with EAAT2 being highly expressed in glia, but not in neurons, and EAAT3 being mostly expressed in neurons. These results are in accordance with previous studies that have shown that EAAT1 and EAAT2 are predominantly expressed in astroglial cells^{14, 97}, while EAAT3 and EAAT4 are expressed in neurons^{98, 99}.

Acute 10 μM and 50 μM L-glutamate insults in bilaminar cultures containing the glial feeder layer did not significantly alter neuronal survival compared to vehicle-treated cultures (not shown). This suggests that the glia play a crucial role in maintaining glutamate at physiological concentrations, most likely through glutamate transporter-mediated clearance. Therefore, we used an acute 100 μM L-glutamate insult for the following experiments, as this glutamate concentration produced significant neurotoxicity and cell death in the presence of glia. Neuronal survival in cultures treated with 10 nM GT949 or GT996 alone was not significantly altered, suggesting that these compounds are not neurotoxic in the presence of glia (figure 1). On the other hand, acute 100 μM glutamate insult significantly decreased neuronal survival to $55 \pm 12\%$ of baseline vehicle levels (figure 1, black bar). Application of 10 nM GT949 after acute glutamate insult resulted in a significant neuroprotective effect, increasing survival levels to $87 \pm 15\%$ of baseline, while the inactive analog GT996 did not increase cell survival levels. The positive control AP-V resulted in significant protection from acute glutamate insult, with survival at $93 \pm 6\%$ of baseline. These results suggest a neuroprotective effect of GT949, possibly due to its mechanism of increasing EAAT2-mediated glutamate uptake.

Neuronal survival after acute glutamate insult in the absence of glia and GT949 treatment—The bi-laminar culture approach allows for separation of the neurons and glia for treatments. We performed an acute L-glutamate insult (10 μM ; 20 minutes) in the absence of glia by transferring neuronal coverslips into glia-free culture dishes containing glia-conditioned medium with L-glutamate. After the acute glutamate insult, neuronal coverslips were returned to their original bilaminar culture dishes, and treated with either GT949 or other control compounds for 24 hours. Then, neuronal coverslips were fixed and immunostained against MAP-2 and imaged by fluorescence microscopy (figure 2A) for downstream survival analysis. At baseline conditions (no glutamate insult or compound treatment - vehicle), neurons did not display pyknotic nuclei or neurite damage, which are indicators of neuronal death^{94, 100}. Neurons treated with GT949 alone (10 nM; 24 hours)

also resemble the baseline vehicle conditions, suggesting that GT949 is not neurotoxic. After acute glutamate insult, we observed reduced MAP-2 staining and DAPI positive, MAP-2 negative cells, indicators of cell death. On the other hand, neurons subjected to acute glutamate insult followed by GT949 (10 nM; 24 hours) displayed morphological characteristics similar to baseline vehicle conditions, suggesting that this compound is neuroprotective in an excitotoxic environment.

Total neuronal survival levels from different experimental conditions were then quantified (figure 2B). Similarly to figure 1, in the absence of glutamate insult, neuronal survival was not significantly altered by 10 nM GT949 or GT996, an inactive analog of GT949, suggesting that these compounds are not neurotoxic at the examined doses. On the other hand, acute, 20 minute insult with 50 and 100 μ M glutamate in the absence of glia resulted in profound neuronal death (not shown) so these concentrations were not used in this experiment. Acute 10 μ M glutamate insult significantly decreased neuronal survival to $38\pm 7\%$ of baseline, vehicle-treated cultures (figure 3B, black bar), and this concentration was used to assess the neuroprotective potential of GT949. Neuronal cultures treated with GT949 for 24h after acute glutamate insult displayed survival of $83\pm 3\%$ of control, a significant increase that suggests a neuroprotective effect of GT949. Cultures treated with GT996, the inactive analog of GT949, after acute glutamate insult displayed survival of $36\pm 6\%$ of baseline, which is not significantly different than glutamate alone. Cultures treated with the NMDA receptor antagonist AP-5 (100 μ M; 24 hours) provides a survival benefit similar to that of GT949 suggesting that excessive glutamate transmission through the NMDA receptor plays a crucial role in glutamate excitotoxicity and neuronal death, thus providing a positive control to measure the effect of GT949. This suggests that NMDA receptor plays a crucial role in glutamate excitotoxicity and neuronal death, likely through activation of neuronal death pathways such as calpain^{101, 102}.

Neuronal survival rates after acute glutamate insult in mixed neuron-glia

cultures—We also investigated the potential neuroprotective properties of GT949 in mixed neuron-glia cultures, a system that more closely resembles physiological conditions in the CNS. We first optimized growth conditions to result in ~15% of glia growth in the cultures after 14 DIV (supplemental figure 3). At 14 DIV, several reports show that mixed cortical neuron-glia cultures express glutamate transporters GLAST/EAAT1, GLT1/EAAT2 and EAAC1/EAAT3^{103–105}.

Then, acute glutamate insults were performed by application of 10, 50, 100 and 1000 μ M L-glutamate for 20 min. However, these insults did not result in significant levels of cell death (not shown), likely because the presence of glia conferred neuroprotection against these insults. Therefore, the neuroprotective efficacy of GT949 was evaluated only in prolonged, 24 hour glutamate insult experiments in the mixed neuron-glia cultures.

Lack of neuroprotective effects of GT949 in primary cultures subjected to H₂O₂-mediated oxidative stress

—We expanded our analysis to test the neuroprotective potential of GT949 in an oxidative stress model in the bilaminar co-culture system. Oxidative stress promotes neuronal death by impairing mitochondrial bioenergetics function and may open the mitochondrial permeability transition pore^{106, 107}. This results in reactive

oxygen species production, energy failure, and release of pro-apoptotic factors such as cytochrome C into the cytoplasm, and damaged to nucleic acids, proteins and lipids¹⁰⁸.

In these studies, bilaminar cultures were exposed to 10 μM H_2O_2 for 20 minutes in the presence of glia, followed by treatment with GT949, or other compounds for 24 hours. As before, neurons were fixed and immunostained against MAP-2 for downstream survival analysis (figure 2).

An acute H_2O_2 insult significantly decreased neuronal survival levels to $36 \pm 13\%$ of baseline (figure 3, black bar). Following the acute H_2O_2 insult with 10 nM GT949 treatment did not significantly improve neuronal survival, as the survival percentage was $35 \pm 6\%$ of baseline. However, blocking NMDA receptors after H_2O_2 insult with 100 μM AP-V significantly increased neuronal survival to $77 \pm 19\%$ of baseline, suggesting that glutamate does play a role in oxidative stress-induced neuronal damage.

Lack of neuroprotective effects of GT949 in mixed neuron-glia cultures subjected to H_2O_2 -mediated oxidative stress

—To further investigate whether compound GT949 had neuroprotective properties in an oxidative stress model, we next subjected 14 DIV mixed neuron-glia cultures to 100 mM H_2O_2 insults for 20 min, and then applied several concentrations of GT949 and 100 mM AP-V for 24 hours. Cultures were then cultures were fixed and analyzed as described before for downstream survival analysis. At baseline conditions (no insult or compound treatment - vehicle), neurons did not display pyknotic nuclei or neurite damage, similar to the observations in bilaminar cultures under control conditions (figure 2A). After acute H_2O_2 insult, we observed reduced MAP-2 staining and DAPI positive, MAP-2 negative cells, indicators of cell death. After insult and application of 1 μM GT949 for 24 hours, we observe cell death characteristics, like the insult alone, indicating lack of neuroprotective effect of the compound. On the other hand, neurons subjected to acute H_2O_2 insult followed by AP-V (100 μM ; 24 hours) displayed morphological characteristics similar to baseline vehicle conditions.

Total neuronal survival levels from different experimental conditions were then quantified (figure 2B). Acute 20 minute insult with 100 μM H_2O_2 resulted in significant neuronal death ($35 \pm 14\%$ of baseline, red bar), and treatments with 100 nM and 1 μM GT949 for 24 hours resulted in survival of $41 \pm 10\%$ and $35 \pm 22\%$ of baseline, which were not significantly different than insult alone. On the other hand, cultures treated AP-V (100 μM ; 24 hours) provides a survival benefit of $102 \pm 19.9\%$ of control, suggesting that glutamate does play a role in oxidative stress-induced neuronal damage.

We also analyzed the effect of the oxidative stress insult on EAAT2 in these cultures via western blot. 14 DIV mixed neuron-glia cultures were subjected to H_2O_2 insults for 20 min, media was changed to fresh media and incubated for 24 hours, then samples were collected for western blotting. Previous studies showed that under conditions of oxidative stress, the EAAT2 transporter would be cleaved and the appearance of a small band around 30 kDa appeared¹⁰⁹. Figure 4C shows a representative western blots of samples ran in triplicate (controls, 10, 100 and 500 mM H_2O_2) that suggests that the transporter is cleaved at higher concentrations of H_2O_2 (100 μM and 500 μM), as we observed a band at around 30 kDa. This

suggests that the lack of neuroprotective effects of GT949 could be due to the H₂O₂ insults resulting in damage of EAAT2.

Our results are in accordance with previous reports showing that oxidative stress increases extracellular glutamate levels, resulting in Ca²⁺ over-load in cultured neurons.^{110, 111} This is followed by activation of MAPK cascades (ERK and/or p38) that induce neuronal cell death¹¹². In the presence of AP-V, neuronal death was mitigated, suggesting that oxidative stress-induced excitotoxicity contributes to neuronal cell death¹¹³. We hypothesize that the lack of efficacy of GT949 under oxidative stress-induced excitotoxicity could be explained by evidence that caspase-3, a key proteolytic enzyme executioner of apoptosis that is triggered by oxidative stress, cleaves EAAT2 at a single site (D505) and decreases glutamate uptake activity^{109, 114}.

However, one study¹¹⁵ shows no neuroprotection in the presence of AP-V, arguing that the H₂O₂-induced neuronal toxicity was not a function of calcium influx into neurons. Several differences in their methodology, as they performed their assays in hippocampal slices and used a higher concentration of H₂O₂, as compared to ours, could at least partially explain the different results. Further investigation is required to understand the cross talk between oxidative stress-mediated neuronal death and NMDA-mediated neuronal death in the context of excitotoxicity.

Effects of acute H₂O₂-mediated oxidative stress in pure glia cultures—

Furthermore, lack of GT949 efficacy may also be explained by previous work suggesting that oxidative stress causes an imbalance in the mitochondrial permeability transition pore (MPTP) of astrocytes, leading to massive glial cell death¹¹⁵. In agreement with this, we have subjected pure glia cultures to insults with increasing concentrations of H₂O₂, where we demonstrate a dose dependent decrease in GFAP+ cells (Figure 5), suggesting that the lack of effect of GT949 in this model is due to damage to the glia, and fragmentation of EAAT2 (seen in figure 4).

Neuroprotective effects of GT949 in primary cultures subjected to prolonged glutamate-mediated excitotoxicity insults

Neuronal survival after prolonged glutamate insult and GT949 treatment in bilaminar cultures—Since we have shown a neuroprotective effect of GT949 after an acute glutamate insult, we next determined if GT949 is also protective during a prolonged, 24 hour glutamate insult. These experiments were all performed in the presence of glia, as neurons in bilaminar cultures do not survive for an extended duration without the glial feeder layer. We began by co-treating 14 DIV bilaminar cultures with 10 μM L-glutamate and a compound of interest for 24 hours, followed by fixation and MAP-2 immunostaining as in previous experiments. As before, neuronal survival was quantified based on MAP-2 staining (figure 6). In the absence of a glutamate insult, 100 nM GT949 or GT996 treatment for 24h did not alter neuronal survival compared to the vehicle-treated baseline, suggesting that the increased dose of these compounds does not induce neurotoxicity. Cultures subjected to prolonged glutamate insult displayed significantly reduced survival, at 49±7% of baseline (figure 6, black bar). Neuronal survival was significantly increased to 80±13%

and to $98 \pm 6\%$ of baseline in cultures co-treated with glutamate and 10 nM or 100 nM GT949. Additionally, 100 nM GT949 produced a more significant effect on survival ($p < 0.01$, compared to insult), suggesting a dose response effect of the compound. In bilaminar cultures treated with glutamate and the inactive analog GT996, survival was $40 \pm 16\%$ of baseline, which was not significantly different from glutamate alone. These results suggest that GT949 is also capable of protecting neurons in a sustained excitotoxic environment, likely by increasing EAAT2-mediated uptake of glutamate.

Neuronal survival and dendritic arborization after prolonged glutamate insult and GT949 treatment in mixed neuron-glia cultures—

Since neurons and glia are grown on different layers in bilaminar cultures, we also wanted to examine GT949 neuroprotection in mixed neuron-glia cultures, where both cell types are in physical contact on the same layer. In these experiments, we evaluated neuronal survival with immunocytochemistry and MAP-2 ELISAs.

Mixed cultures in each treatment group were stained against the neuronal marker MAP-2 (green), the astrocyte marker GFAP (red), and the nuclear marker Hoechst (blue) (figure 7A). As in bilaminar cultures, mixed cultures with no glutamate insult or compound treatment (vehicle), as well as cultures treated with GT949 alone do not show damaged neurites or nuclei. Mixed cultures subjected to a prolonged 10 μM glutamate insult show neuronal loss, which is rescued by co-application of 10 nM GT949, suggesting a neuroprotective effect of the compound in these cultures.

Next, neuronal survival was quantified in each treatment group using the immunostaining method (figure 7B). In the absence of glutamate, neuronal survival was not significantly altered after 24h incubation of 100 nM GT949 or GT996, or 100 μM DL-threo- β -benzyloxyaspartic acid (TBOA), a glutamate transport inhibitor, suggesting that these compounds are not neurotoxic at their respective doses. Cultures subjected to a 10 μM glutamate insult for 24h displayed significantly reduced neuronal survival, to $50 \pm 11\%$ of baseline (figure 7B, black bar). However, co-treatment with 10 or 100 nM GT949 significantly increased survival levels to $78 \pm 11\%$ and $97 \pm 31\%$ of baseline respectively, suggesting a neuroprotective dose response effect of GT949. To determine if the neuroprotective effect of GT949 requires an active glutamate transporter, we subjected mixed cultures to prolonged glutamate insult, followed by co-treatment with 100 nM GT949 and the glutamate transport inhibitor TBOA (100 μM), that inhibits all transporter subtypes at this concentration, and with the selective EAAT2 inhibitor WAY 213613 (1 μM). Neuronal survival in these cultures was $54 \pm 9\%$ of baseline in presence of TBOA, and $15 \pm 9\%$ in presence of WAY 213613, which were not significantly different from glutamate alone, and suggests that the neuroprotective effect of GT949 requires active glutamate transporters, and specifically, EAAT2 activity. As expected, co-treatment with glutamate and the inactive analog GT996 (10 μM) did not affect survival ($31 \pm 5\%$ of baseline), while co-treatment with glutamate and the NMDA receptor antagonist AP-5 (100 μM) significantly increased neuronal survival to $94 \pm 6\%$ of baseline.

As a second measure for neuroprotection, quantification of dendritic arborization using Sholl analysis¹¹⁶ was performed, which includes counting the number of dendritic intersections

that occur at fixed distances from the soma in concentric circles. This analysis reveals the number of branches, branch geometry, and overall branching patterns of neurons¹¹⁷. In this way, we evaluated changes to the dendritic arbor as a whole, after prolonged glutamate-mediated insults and GT949 treatment in mixed neuron-glia cultures.

The average dendrite length and dendrite number was decreased in response to glutamate insult (figure 7C). These results are in accordance to previous literature showing that glutamate-mediated excitotoxicity results in significant reduction in total dendrite output, which was accompanied by significant decrease in primary dendrite number and individual primary dendrite length¹¹⁸. Subsequent treatment with GT949 has no effect on restoring dendritic length but does restore dendritic number, as there are no significant differences between insult alone and insult with co-application of GT949. Additionally, treatment with 100 nM GT949 alone increases the number of dendrites.

We also performed MAP-2 based ELISAs as a surrogate approach to measure neuronal survival in mixed cultures (figure 8). A prolonged 100 μ M glutamate insult significantly decreased MAP-2 expression to $43 \pm 11\%$ of baseline levels (figure 8, black bar), while co-treatment with 10 nM GT949 significantly increased MAP-2 expression to $86 \pm 27\%$ of baseline. Co-treatment with 10 nM GT996 resulted in survival at $52 \pm 19\%$ of baseline, which was not significantly different from glutamate alone. These results demonstrate that GT949 preserves neuronal survival and rescues expression of MAP-2 in cultures exposed to high levels of glutamate for an extended duration.

In summary, we established that GT949 was neuroprotective after acute and prolonged glutamate insults in two different types of cortical neuron-glia cultures. This neuroprotective effect disappears when co-incubated with selective EAAT2 inhibitor WAY 213613 (figure 7B), indicating that the mechanism of GT949 clearly involves an active EAAT2 transporter. This suggests that increased glutamate clearance via EAAT2 activation can prevent excitotoxic damage and cell death.

The level of insult (concentration and time) results in varied levels of neuronal death, depending on the type of culture. In the bi-laminar system, the acute glutamate insult performed in the presence of glia (figure 1) required 10x higher concentrations of glutamate than in the absence of glia (figure 2) (i.e., 100 μ M versus 10 μ M) to result in the same level of neuronal death (~50%). This suggests a critical role of the glia in maintaining glutamate at physiological concentrations, most likely through glutamate transporter-mediated clearance. The same pattern was observed in the oxidative stress insults, when comparing the bilaminar system (figure 3), and in the mixed neuron-glia cultures (figure 4). The level of H₂O₂ insult had to be 10x higher in the mixed culture to reach the same level of insult as in the bilaminar culture. In the mixed neuron-glia cultures, intrinsic connections of the two different cell populations growing in the same layer makes this type of culture more physiological than the bi-laminar approach, where the two cell populations grow in different layers. However, we are very encouraged by the fact that we observe neuroprotective properties of GT949 in both approaches.

Strikingly, in the mixed neuron-glia culture system with ~15% of glia (supplemental figure 3) we were able to show neuroprotective properties of GT949 (figures 4, 7–8), suggesting that the neuroprotective effects of this class of compounds *in vivo*, where the proportion of glia to neurons is at least 1:1 and has been speculated to be as high as 10:1¹¹⁹, would be amplified.

Changes in dendritic arborization is as an important caveat as neuronal function is reliant upon synaptic connection. In this work, we observed that a glutamate insult decreased the dendrite length and number, in accordance to previous reports. There is good evidence that changes in dendritic and axonal morphology and properties take place in neurodegenerative disease in advance of neuronal loss^{118, 120}. Subsequent treatment with GT949 showed a trend on increasing dendritic number. Additionally, treatment with 100 nM GT949 alone seems to have a beneficial effect on dendritic number, suggesting that GT949, in addition to rescue neuronal death, has effects on dendritic arborization and could modulate synaptic connection. Future studies are needed to further understand whether GT949 could function as a neuroprotective approach against progressive dendritic loss.

In this study, we subjected cultures to acute (20 min) and prolonged (24 hours) excitotoxic insults. Glutamate excitotoxicity plays a key role in secondary damage following acute pathologies, including TBI, epileptic seizures and stroke. Therefore, we propose that a compound like GT949, that has neuroprotective effects after acute insults *in vitro*, could be developed for the treatment of these pathologies, as long as the compound is administered within the window of opportunity, as the peak of glutamate excitotoxicity is known to be of short duration following the insults^{57, 121}. Development of therapies for these pathologies is highly sought after, as, to date, no compelling efficacy data have been published regarding any pharmacological therapies for TBI and stroke¹²².

On the other hand, some CNS disorders are characterized by chronic excitotoxic events, such as ALS and Alzheimer's disease^{46, 123}. Because we observed a neuroprotective effect of GT949 after prolonged (24 hours) excitotoxic insults in cultures, we can also propose the development of a therapy for such disorders based on this compound. However, it is also known that the level of glutamate transporters can be downregulated in these chronic pathologies^{13, 124}, therefore it is likely to be challenging that allosteric modulation of the glutamate transporter to be effective. In this sense, the use of transport expression enhancers (such as ceftriaxone^{37, 67–70} and clavulanic acid^{125–127}) might be beneficial to restore transporter levels, and GT949 could be then used as adjuvant therapy to enhance transport activity levels and increase glutamate clearance.

The glutamate and oxidative stress insult investigated in this study are implicated in a wide range of CNS disorders, such as acute disorders like TBI¹²⁸, epilepsy¹²⁹ and chronic neurodegenerative disorders, like Alzheimer's, Parkinson's and Huntington's, that involve neurotoxic aggregation of specific proteins in the brain¹³⁰. Although the relationship between oxidative stress and development of protein aggregates is not completely established, it is speculated that simultaneously occurring glutamate toxicity, calcium imbalance, and mitochondrial impairment collectively intensify oxidative stress¹³⁰. This cycle of biochemical distress in the brain might suggest that targeting the antioxidant

defense may offer a better potential to limit these pathologies, rather than focusing on targeting glutamate excitotoxicity. On the other hand, some studies suggest a vicious cycle in neurodegeneration that includes glutamate excitotoxicity, oxidative stress, and mitochondrial dynamics. Therefore, possible neuroprotective strategies might include blockade of NMDA receptor activation, enhancement of SOD2 expression to clear mitochondrial ROS (reactive oxygen species), and inhibition of the downstream apoptotic cascade and positive modulation of glutamate transport^{46, 131, 132}. Likewise, one might propose that intervention by preventing excitotoxicity through increased glutamate transport function could also break this cycle, as averting excitotoxicity would result in limiting the production of ROS if the treatment is offered at an appropriate time window. Determining the best time window for compound administration is out of the scope of these studies, as additional preclinical studies, drug development efforts and clinical trials are necessary to accurately determine this parameter. However, we suggest that the use of multiple therapies targeting these different components of the diseases' pathology might offer potential beneficial pharmacological therapies in the future.

In this work, we focused on describing the neuroprotective effect of GT949 as associated to its actions on glutamate transporter EAAT2, but we also recognize the possibility that other mechanisms of neuroprotection could be involved. For instance, metabotropic glutamate receptors (mGluRs) can exert different actions (toxic or compensatory) in response to excitotoxic stimuli¹³³.

Activation of group I mGluRs, which are located mostly on postsynaptic neurons, increase NMDA receptor activity and risk of excitotoxicity. Activation with agonist DHPG results in exacerbation of ischemic brain damage¹³⁴, and agonist 1S,3R-ACPD induces cell death in rat striatum¹³⁵. On the other hand, antagonist LY 367385 modulates presynaptic release, resulting in neuroprotection¹³⁶. Activation of group II and III mGluRs, localized mostly in the presynapsis, decrease NMDA receptor activity and risk of excitotoxicity. For example, activation with selective agonists DCG-IV or L-AP4 inhibited apoptosis of primary cultured mesencephalic neurons¹³⁷, and exerted neuroprotection on astrocytes via promoting glutamate uptake^{137, 138}, and antagonists APICA or MSOP completely abolished their neuroprotective effects¹³⁷. Acute or chronic exposure of astrocytes with selected mGluR II agonists attenuate glutamate release¹³⁹. Moreover, glutamate transporter protein expression was shown to be regulated after stimulation with selected mGluR agonists in cultured human glial cells¹⁴⁰, and conversely, there is evidence that astrocyte glutamate transporters regulate mGluR1-mediated excitation of hippocampal interneurons¹⁴¹. Collectively, these studies suggest that mGluRs are modulated by excitotoxic insults and can act as compensatory mechanism responding to elevated extracellular glutamate levels. Future studies will determine whether mGluRs modulation is involved in the action of GT949 on transporters.

Future studies will also investigate cell death mechanism in these models, if apoptotic in nature, i.e., mediated by caspases or calpains¹⁴²; or necrotic¹⁴³. Additionally, will also investigate intracellular pathways that play key roles after excitotoxic insults and treatments with glutamate transporter allosteric modulators. Possible pathways include ERK/12, that plays a crucial role in promoting cell death in neurodegenerative diseases¹⁴⁴; p38, a potent regulator of stress-induced apoptosis¹¹²; Akt, one of the central kinases associated with

mediating survival signaling¹⁴⁵; and NF- κ B, which ameliorates disease if inhibited in glia, and may enhance memory in neurons¹⁴⁶.

CONCLUSIONS

In summary, our results suggest that this class of EAAT2 PAM compounds may be clinically useful for acute and chronic disorders in which glutamate excitotoxicity occurs, such as stroke, TBI and epilepsy, among others. However, we recognize that a combination of drugs targeting various aspects of neuroprotection, neuroinflammation and regeneration may be needed, as these are complex pathologies^{22, 147–149}. Our results also suggest that we could avoid side effects that are typically associated with glutamate receptor antagonism, as our EAAT2 PAMs do not modulate the function of NMDA receptors⁸⁹.

We aim to build from these initial studies by measuring glutamate concentration in culture media after insult and compound treatments, and determining the mechanism of cell death in our model of acute and prolonged glutamate insults. Additionally, we aim to determine the neuroprotective efficacy of GT949 and related compounds in other models of *in vitro* neurotoxicity, including NMDA-mediated insults, stretch injury (*in vitro* TBI^{150, 151}), Mg²⁺ deprivation (*in vitro* epilepsy¹⁵²) and oxygen-glucose deprivation (*in vitro* stroke^{36, 153}). Further efforts will focus on making improved GT949 analogs with suitable drug-like properties and adequate pharmacokinetic properties for progression to *in vivo* studies, such as brain injury models. This will include determining the route of administration, therapeutic window, and safety in combination with other long-term treatment strategies.

Safe and effective pharmacological approaches to treat patients after excitotoxic events such as brain trauma are urgently needed^{121, 142, 154}. Importantly, our EAAT2 PAM compounds provide a starting point for identification of a new class of neuroprotective compounds that function by enhancing the removal of excessive glutamate in the synaptic cleft and preventing glutamate-mediated excitotoxicity.^{36, 83, 155–157}

MATERIALS AND METHODS

Compounds

The identification of allosteric modulators (AMs) of glutamate transporters has been described previously⁸⁹. Compounds GT949 (positive allosteric modulator of EAAT2) and GT996 (inactive compound, used as negative control) were synthesized by the Ugi multi-component reaction^{158–161} using commercially available starting materials. Compounds were dissolved to 10 mM stocks in Dimethyl sulfoxide (DMSO), the maximum concentration of DMSO in the vehicle experiments was 0.01%.

L-glutamate, (2R)-amino-5-phosphonovaleric acid (AP-V), DL-threo- β -Benzyloxyaspartic acid (TBOA) and WAY 213613 were purchased from Tocris (Bristol, United Kingdom). Hydrogen peroxide solution (H₂O₂) was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture reagents and media

Cell culture media including high-glucose Dulbecco's Modified Eagle Medium (DMEM) and Neurobasal medium with pyruvate, glutamine, gentamicin, HEPES (1 M), B27 and N2 supplements were purchased from Gibco (Waltham, MA, USA).

Poly-L-lysine, ovalbumin, bovine serum albumin, DNase and Cytosine- β -D-arabinofuranoside were purchased from Sigma-Aldrich (St. Louis, MO).

Fetal bovine serum and horse serum were purchase from Hyclone (South Logan, UT, USA).

Poly-lysine coated 96-well plates and phosphate-buffered saline (D-PBS) were purchased from Corning (Corning, NY, USA).

Tools for animal dissection was purchased from Biomedical Research Instruments (Silver Spring, MD, USA).

Antibodies

Primary antibodies for immunocytochemistry included polyclonal anti-microtubule associated protein-2 (MAP-2, cat: AB5622) and monoclonal anti-gial fibrillary acidic protein (GFAP, cat: MAB360), both purchased from Millipore (Temecula, CA, USA).

Secondary antibodies used for immunocytochemistry included Alexa Fluor® 488 conjugated AffiniPure Donkey Anti-Rabbit IgG and CyTM3-conjugated AffiniPure Donkey Anti-Mouse IgG. These antibodies, along with normal donkey serum and normal goat serum, were purchased from Jackson Immuno Research (West Grove, PA, USA).

Antibodies used for ELISA were MAP-2 (monoclonal, cat: 13-1500) and Goat anti-Mouse IgG (H+L) Secondary Antibody (HRP conjugate) from Thermo Fisher Scientific (Waltham, MA, USA).

Primary antibodies for western blotting EAAT1 Rabbit mAb (1:100, cat: 5684), EAAT3 Rabbit mAb (1: 100, cat: 14501) and β -actin mouse mAb (1:1,000), were purchased from Cell Signaling (Danvers, MA, USA). EAAT2 rabbit polyclonal (1: 1,000, cat: AGC-022) was purchased from Alomone, Jerusalem, Israel. Infrared-conjugated secondary antibodies [(Anti-rabbit DyLight 800 (Green) and Anti-mouse DyLight 680 (Red), both at 1:1,000)] were used for imaging using LI-COR Image Studio, version 4.0.21 (LI-COR Biosciences; Lincoln, NE, USA).

Animals

HsdHot: Holtzmann rats were purchased from Charles River (Malvern, PA, USA), and housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities. Animal housing and experimental procedures were also approved by the Drexel University College of Medicine Institutional Animal Care and Use Committees (IACUC).

Primary cultures

These studies utilized two different *in vitro* primary neuronal cultures: a bilaminar co-culture of neurons and glia, in which neurons and glia grow in different layers; and mixed neuron-glia cultures, in which these cells grow together in the same layer.

Bilaminar co-culture of primary rat cortical neurons and glia—Bilaminar cultures were prepared as previously described^{91, 92, 162}. Briefly, cortical neurons were obtained from late embryonic stage (E17) Holtzman rats. For immunocytochemistry, neurons were plated on poly-L-lysine-coated glass coverslips (15-mm diameter) at a density of 35,000 cells/coverslip. After 3 hours in plating medium, 3 to 5 neuronal coverslips were transferred to a single 60 mm culture dish containing an astroglia-enriched secondary culture of mixed glia. Glial cultures were prepared using cortices of newborn Holtzman rat pups (P2–4) prior to the neuronal dissection, as previously reported¹⁶³. For protein isolation and western blots, neurons were plated in 60mm dishes at a density of 1×10^6 cells/dish. Primary glia plated on Thermanox sheets were added to the neuronal culture dish 4 hours after plating, facing the neuronal layer⁹².

Cytosine arabinofuranoside (AraC) was added to the cultures 24–48 h after plating to halt non-neuronal cell proliferation (2 μ M for cultures with neuronal coverslips, and 10 μ M for cultures with glial Thermanox coverslips). The bilaminar culture was grown for 14 DIV in a 37°C incubator (5 to 10% CO₂). On day 7, 25% of the media was replaced by fresh media. The osmolarity of the culture media was assessed every 5 days, and adjusted to 300–310 mOsm if necessary. In parallel, 60 mm dishes containing only the astrocyte-enriched layer were cultured as above to generate conditioned media for further use.

In this preparation, ~97% of viable cells in these cultures are neurons (Figure 1 of supplemental material).

Mixed neuron-glia cultures—As a second model for validation of the effect of the compounds, we used mixed neuron-glia cultures, in which neurons and glia cells are grown in the same layer. Cortical cells were obtained from late embryonic stage (E17) Holtzman rats. These cells were plated at a density of 35,000 cells/coverslip in 12-well plates for immunocytochemistry assays, and 10,000 cells/well in poly-D-lysine pre-coated 96-well plates for ELISA assays, as previously described^{164–166}, with modifications.

We did not add AraC to mixed neuron-glia cultures. Culture conditions were optimized to contain enough glia to observe suitable expression of EAAT2 and treatment effects with GT949, while maintaining a population of neurons that is large enough to accurately measure neuronal survival. This was achieved by plating cells in Neurobasal media (NB) with 2% B27 supplement and 10% Horse Serum. The media was changed 2 h after plating to NB media containing 2% B27, 2% Horse serum, 1% glutamine, 25 μ M glutamate and 50 μ g/mL gentamicin. 4 days later, media was changed to NB media with 2% B27, 1% glutamine and 50 μ g/mL gentamicin. Finally, cells received fresh NB media (with 2% B27, 1% glutamine and 50 μ g/mL gentamicin) at 8 DIV, and cultures were exposed to excitotoxic insults and compound treatment at 14 DIV. These growth conditions produced approximately 15% glia in each culture (supplemental figure 3). This level of glia allowed

for sufficient EAAT2 expression to observe an effect of GT949, while maintaining a large enough population of neurons for the quantification of MAP-2 in culture. Only cultures with consistent percentages of astrocytic populations across individual experiments and coverslips were considered. Any culture with a proportion of glia that was significantly different from a range of 7–18% (measured by One-Way ANOVA followed by Newman-Keuls posthoc test for multiple comparisons performed on the percentage of GFAP+ in each coverslip) was excluded from the analysis.

Western Blotting

Expression of glutamate transporters EAAT1–3 were analyzed in both glia and neurons from bilaminar cultures. Cell lysates were prepared from secondary glia or primary neurons grown in 60mm dishes for 14 DIV. After lysis, collection and centrifugation, protein quantification was performed by using a Pierce BCA Protein Assay Kit. Samples (40 µg protein/10µL/lane) were subjected to SDS-PAGE using NuPAGE 4–12% Bis-Tris Mini Gels. Primary antibodies for EAAT1–3 are listed above, which were incubated overnight on a rocker at 4°C. The following day, primary antibodies were removed and membranes were washed 3 times at 10 min each with PBS containing 0.05% Tween (PBS-T). After the final wash, infrared-conjugated secondary antibodies (listed above) were added and blots were placed on the rocker for 60 min at room temperature. After 3 additional washes with PBS-T, imaging and band density measurements were performed using the Odyssey imaging system (LI-COR Image Studio, version 4.0.21)."

Additionally, mixed neuron-glia cultures were plated on 6 well plates, and at 14 DIV exposed to oxidative stress insults by incubation with 10, 100 and 500 µM H₂O₂ for 20 min, and 24 hours later subjected to SDS- PAGE and probed with EAAT2 and actin antibodies, as described above.

***In vitro* excitotoxic insults (glutamate and oxidative stress) and compound administration**

All insults were performed in well-differentiated cultures (14 DIV), to ensure that protein content and mitochondrial respiration were stabilized^{104, 167}. A report shows that 14 DIV cultures also express markers of neuronal maturity, such as NeuN; NF-L; NF-M; GAD; TH; PSD-95 and synaptophysin¹⁶⁸. All cultures were examined before each experiment for correct osmolarity and cell viability under a TCM-BR inverted brightfield microscope. Cultures that appeared unhealthy were discarded from further experiments. Insult solutions for bilaminar cultures were prepared in glia-conditioned media (collected from a glia feeder), to assure that neurons were supplied with all required factors throughout the experiment. Glutamate and compound solutions were made in fresh growth media specific for each culture.

Cultures were subjected to three types of insults, as described below.

Acute glutamate insults—Cell cultures were exposed to glutamate (10 or 100 µM), or vehicle for 20 minutes. Minor modifications were performed according to type of culture, as follows:

- i.** Bilaminar cultures in the absence of glia: neuronal coverslips were separated from the glial feeder layer and transferred to dishes containing insult solutions (10, 50 and 100 μM L-glutamate or vehicle) for 20 min. Subsequently, coverslips were returned to their original culture dishes containing the glial feeder layer and either 10 nM or 100 nM GT949 (PAM of EAAT2), GT996 (inactive analog, used as negative control), 100 μM AP-V (NMDA receptor antagonist, used as positive control) or vehicle, which were subsequently incubated for 24h until neuronal survival analysis. Previous results indicated that GT949 had an EC_{50} of $\sim 1 \text{ nM}$ ⁸⁹ for the effect on glutamate transport augmentation, so we tested the compound at 10 and 100X higher concentrations to ensure that the transporters were fully stimulated.
- ii.** Bilaminar cultures in the presence of glia: conditioned media containing the insult solutions (L-glutamate at 10, 50 or 100 μM) or vehicle were added to dishes with neuronal coverslips and glial feeding layer. After incubation for 20 min, solutions were replaced with conditioned media containing compounds GT949, GT996, AP-V (at the same concentrations as above) or vehicle. Cultures were incubated for 24 hours, followed by downstream analyses.
- iii.** Mixed neuron-glia cultures: L-glutamate (100 μM and 1mM) were directly added to the mixed cultures for 20 min. Since these insults did not result in significant levels of neuronal death (not shown), these assays were excluded from this study.

Prolonged glutamate insults—Cultures were exposed to L-glutamate (10 or 100 μM , or vehicle), for 24h. Minor modifications were performed according to type of culture, as follows:

Bilaminar cultures: cultures containing neuronal coverslips and the glial feeder layer were randomly exposed to L-glutamate (10 μM or 100 μM), or vehicle in the presence of compounds (10 or 100 nM of GT949 or 100 nM GT996) or vehicle, prepared in conditioned media, for 24h.

Mixed neuron-glia cultures: L-glutamate (10 or 100 μM) or vehicle was directly added to mixed cultures. Compounds GT949 or GT996 (10 nM or 100 nM), or AP-V (100 μM), were co-applied and allowed to incubate for 24h. As we also aimed to validate that the GT949 neuroprotective effect was mediated by modulation of glutamate transport, we also co-applied glutamate transport inhibitor TBOA (100 μM) and selective EAAT2 inhibitor WAY 213613 (1 μM) for 24 hours.

Additionally, to test for any possible toxicity of the experimental compounds, cultures were treated with only GT949 or GT996 (100 nM), for 24 hours.

Oxidative stress insults model of excitotoxicity—To mimic an oxidative stress model of excitotoxicity¹¹¹, H_2O_2 was applied to bilaminar cultures in presence of glia feeder or mixed neuron-glia cultures for 20 min, as described above. Briefly, H_2O_2 (10 μM , 50 μM or 100 μM , for bilaminar and 100 μM for mixed neuron-glia cultures) prepared in conditioned media was added for 20 min, then removed, and compounds and positive controls were added and cells incubated for 24 hours.

Acute insult with H₂O₂ performed in the absence of the glia-feeding layer and prolonged insults in presence of the glia-feeding layer resulted in high levels of neuronal death (data not shown); therefore, these assays were excluded from this study.

Immunocytochemistry assays for evaluation of neuronal survival

To evaluate neuronal health status (as a function of MAP-2) and the effect of treatment with the compounds, cultures on 15 mm glass coverslips (from both bilaminar and mixed approaches) were fixed and fluorescently labeled as described^{90, 169, 170}. As a control for initial levels of neuronal survival, neurons not subjected to treatments (time zero) were also examined. Briefly, cultures on coverslips were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% Normal Donkey serum, washed and incubated overnight with primary antibodies in 5% blocking solution: anti-MAP-2 (1:1,000) and anti-GFAP (1: 400). The following secondary antibodies were used: Alexa Fluor® 488 conjugated AffiniPure Donkey Anti-Rabbit IgG for MAP-2 detection and CyTM3-conjugated AffiniPure Donkey Anti-Mouse IgG for GFAP, both at 1:500, prepared in blocking solution (5% normal donkey serum in PBS). After 30 min incubation in the dark, coverslips were washed and mounted on a slide with ProLong® Diamond Antifade Mountant with DAPI for nuclear staining. Coverslips were then sealed with clear nail polish.

Image acquisition

Cells were imaged with a fluorescent microscope to examine morphology and a confocal microscope to quantify cell survival. For the fluorescent microscope, we used the following settings: quality: 1280×1024 16-bit, 2.4x analog gain, and dynamic contrast. The exposure was adjusted between 400ms and 2s based on which time provided optimal fluorescence. For the confocal microscope, we used the following settings: quality: 1280×1024, 2.4x analog gain, and dynamic contrast. The exposure was set at 8 seconds and images were taken using the 30x zoom setting. For all experiments, 5–10 random fields/coverslip and 3–4 coverslips were evaluated per treatment condition. Images were taken in all 3 filters to visualize DAPI, Alexa-488 (for MAP-2), and CY-3 (for GFAP). For assessment of cell population, cells in the cultures were classified into neurons (MAP-2 positive cells) and glia (GFAP positive cells). The abundance of each cell type was calculated and expressed as percent of total, DAPI positive cells on a coverslip. In bilaminar cultures, ~97% of cells in neuronal layers were neurons, but a small percentage of glia and other cells were identified (DAPI positive, MAP-2 negative and GFAP negative) (supplemental figure 1). For mixed neuron-glia cultures, roughly 70% of cells were neurons, and 15% of the cells were glia (supplemental figure 3).

Neuronal survival counting and experimental blinding

Neuronal survival was evaluated by individuals blinded to the treatment conditions. Survival was determined by evaluating individual neurons for features that are indicative of neurodegeneration and apoptosis, such as pyknotic nuclei, membrane blebbing, and neuritis^{171, 172}. Nuclei that stained with DAPI but lacked significant MAP-2 staining were also classified as dead neurons. The percentage of neuronal survival was calculated by dividing the number of MAP-2 positive neurons with no morphological signs of toxicity, by the total number of MAP-2 positive neurons on each coverslip.

For each experimental condition, 5 random fields from 3–4 coverslips were analyzed, totaling at least 100–150 neurons. Mean survival in each treatment condition was normalized to mean survival in the control group for each experiment. The normalization was performed considering the control group of each individual experiment as 100% of survival and obtaining the relative percentage of neuronal survival of each condition/treatment. Additionally, neuronal survival was evaluated before all insults (time zero) in at least 4 control coverslips, before beginning each experiment. Only sets of experiments with reasonable survival rates at time zero (>75%) were included in the final analysis of neuronal survival.

Images were processed using ImageJ® from the National Institute of Health (Bethesda, MD, USA).

Dendritic arborization analysis

As a second measure for neuroprotection, we quantified arborization, as alterations in dendritic shapes have been shown to be aberrant during disease or injury^{117, 173}. Neurite arborization analysis was performed in mixed neuron-glia cultures subjected to control (no insult) and to acute and prolonged (??) L-glutamate insult (in presence and absence of 100nM GT949) conditions. Images were analyzed using the Neurite Tracer and Scholl Analysis plug-ins for Image-J (NIH, Bethesda, MD). 6–10 single neurons from separate images for each condition were isolated and then traced using the Neurite Tracer plug-in and were then processed by Scholl analysis¹¹⁶ to analyze neurite length and branching.

Immunocytochemistry assays for evaluation of toxicity of compounds and insults in pure glia cultures

To evaluate potential toxicity of compound GT949 and oxidative stress insult in pure astrocyte cultures, glia cultures were plated in coverslips, as described above. After 14 DIV, cultures were incubated with increasing concentrations of GT949 (1µM - 100µM) or H₂O₂ for 24h. Then coverslips were fixed, fluorescently labeled as described above, and subsequent morphological and quantification analysis of GFAP-positive cells were performed.

We also analyzed toxicity of the oxidative stress insult *per se*, as previous literature suggested that this type could result in glial cell death^{109, 114, 115}. Pure glial cultures were subjected to the H₂O₂ insults as described above. Following the 20 min insult, media was changed to fresh glia media and incubated for 24h. Cultures were then fixed and immunocytochemistry was performed to analyze glial morphology and survival as described above.

ELISA assays

Mixed neuron-glia cultures plated in 96-well plates were subjected to cell-based MAP-2 ELISAs to evaluate neuronal survival. Briefly, after insult and compound treatments, plates were washed with phosphate buffered saline (PBS) using a plate washer, and fixed with 4% paraformaldehyde. The cell membranes were then permeabilized with 0.1% Triton-X, and non-specific antibody binding was blocked with 10% normal goat serum. Cells were then

incubated with MAP-2 primary antibody (1:1,000) overnight. Primary antibody was omitted from the top two rows on each plate to allow an evaluation of the background (non-specific antibody binding). After washing, the HRP-conjugated secondary antibody (1:500 in 10% Goat Serum) was incubated for 30 min in at 37°C, and TMB substrate was added to the plates to detect horseradish peroxidase activity, yielding a blue color ($A_{\text{max}} = 370\text{nm}$ and 652nm) that changes to yellow ($A_{\text{max}} = 450\text{nm}$) upon addition of 2M Sulfuric Acid solution. Absorbance was measured with a spectrophotometer at wavelengths of 450nm and 550nm. Data from at least 12 wells per treatment was plotted as a function of absorbance, which correlates to the amount of MAP-2 binding. Background (obtained in absence of the addition of primary antibody) was subtracted from the total.

Data analysis

All data were analyzed using GraphPad Prism®, version 5.0 for PC (GraphPad Software, La Jolla, CA, USA). Graphs represent average \pm SEM of at least three independent experiments performed in triplicate.

Statistical significance was assessed using One-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparisons *posthoc* test with vehicle as control (* $p < 0.05$), or Newman-Keuls *posthoc* test for multiple comparisons (# $p < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Bradley Nash and Lindsay Festa in the Meucci Lab for assistance with primary culture techniques, Bradley Nash for the revision of the manuscript, and Ole. V. Mortensen for helpful discussions (all from Department of Pharmacology and Physiology, Drexel University).

Funding sources

This work was supported by Drexel University's startup funds to ACKF, and NIH grants to OM (DA15014 and DA32444). This project is also funded, in part, under a grant with the Pennsylvania Department of Health to J.M.S. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions. We would also like to acknowledge the Brazilian Scientific mobility program sponsored by CAPES & CNPQ for providing financial support to R.M.F. (Grant ID 15149399).

Abbreviations used:

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-V	(2R)-amino-5-phosphonovaleric acid
AraC	Cytosine- β -D-arabinofuranoside
CNS	Central nervous System

DAPI	4',6-Diamidino-2-Phenylindole
DIV	Days in vitro
DL-TBOA	DL- threo- β -Benzyloxyaspartic acid
EAAT1–5	Excitatory Amino Acid Transporters 1–5
ELISA	Enzyme-Linked Immunosorbent Assay
GFAP	Glial Fibrillary Acidic Protein
GlTPh	<i>Pyrococcus horikoshii</i> glutamate transporter
HRP	horseradish peroxidase
HS	horse serum
MAP-2	Microtubule-Associated Protein 2
NB media	Neurobasal media
NMDA	N-methyl-D-aspartate
OGD	Oxygen-glucose deprivation
PAM	Positive allosteric Modulator
PBS	Phosphate Buffer Saline
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBI	Traumatic Brain Injury
TBOA	DL-threo- β -Benzyloxyaspartic acid
TMB	–3,3',5,5'-Tetramethylbenzidine, chromogenic substrate for HRP detection in ELISA
WAY 213613	N-[4-(2-Bromo-4,5-difluorophenoxy)phenyl]-L-asparagine

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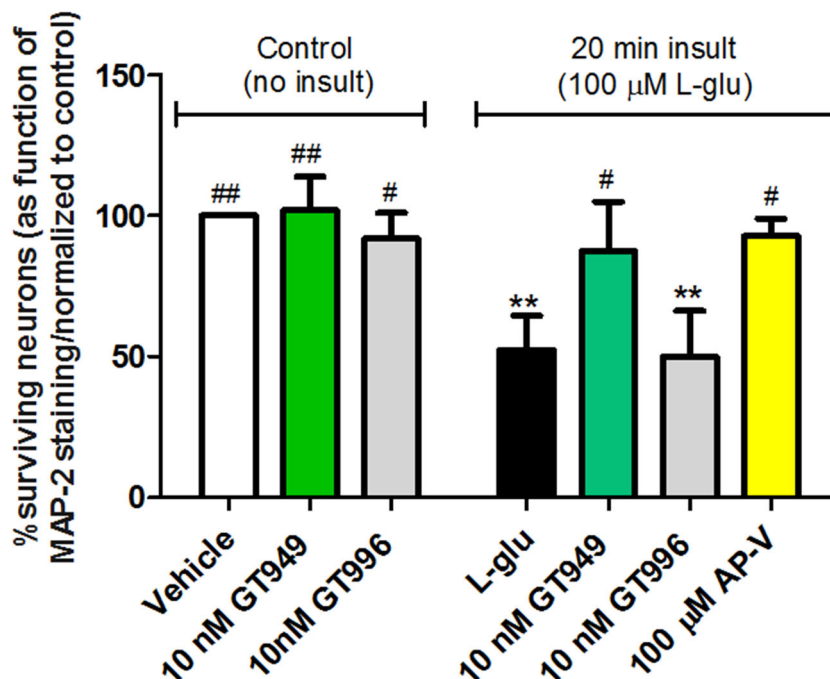


Figure 1. Neuroprotective properties of GT949 in bilaminar cultures after acute exposure to glutamate (in the presence of glia).

Bilaminar cultures at 14 DIV were treated with glia-conditioned medium containing L-glutamate for 20 minutes, followed by replacement of the glutamate-containing medium and compound treatment for 24 hours. Then, cells were fixed and immunostained as previously described for neuronal survival analysis. Application of 10 nM GT949 and GT996 alone had no noticeable effects on neuronal survival. Acute 100 μM L-glutamate significantly reduced neuronal survival, and GT949 and AP-V mitigated L-glutamate toxicity. GT996 also had no effect on L-glutamate toxicity. Data was normalized to control. 3–4 coverslips were assessed per treatment condition and 100–150 cells were manually counted per treatment. Neuronal survival data is representative of 3 independent experiments and control levels were not statistically different for normalization purposes. ** $p < 0.01$, vs. control (no insult), ## $p < 0.01$, # $p < 0.05$, vs. insult.

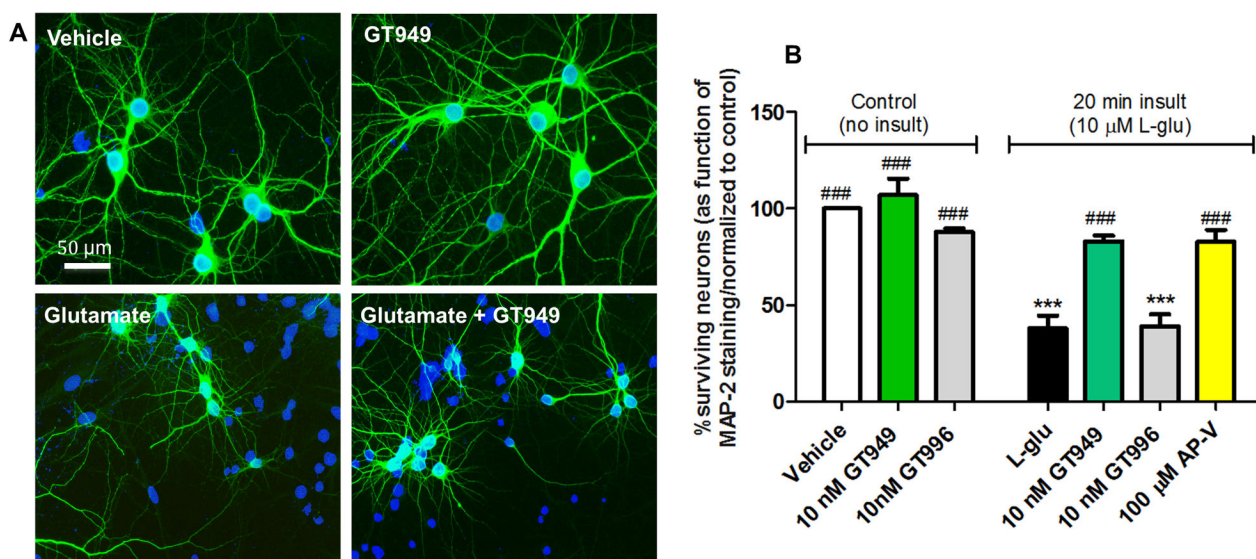


Figure 2. Neuroprotective properties of GT949 in bilaminar cultures after acute exposure to glutamate (in the absence of glia).

A. Representative images of cultures after different treatments in the absence of glia. The neuronal layer of bilaminar cultures was immunostained against MAP-2 (green), and counterstained with the nuclear marker DAPI (blue). Cultures exposed to vehicle, or 100 nM GT949 alone do not display obvious degeneration or cell death. Cultures acutely exposed to L-glutamate (20 min, 10 μ M) show increased DAPI positive, MAP-2 negative cells, indicative of toxicity and neuronal death. However, co-exposure of 10 nM GT949 reversed a portion of the cellular damage caused by L-glutamate. All images are shown at 40x magnification. Scale bar: 50 μ m.

B. Quantification of neuronal survival in the absence of glia. After vehicle or L-glutamate (20 min, 10 μ M) exposure in glia-free conditions, coverslips were transferred back to their original glia dishes in the presence or absence of GT949 or GT996 (10nM) or the NMDA antagonist AP-V (100 μ M) for 24 hours. Then, cells were fixed and immunostained as in A, and analyzed for cell death. GT949 and GT996 alone did not alter neuronal survival levels. Acute L-glutamate significantly decreased neuronal survival, while GT949, and AP-V mitigated L-glutamate neurotoxicity. GT996, the inactive analog, had no effect on neuronal survival after L-glutamate exposure. Data was normalized to control, 3–4 coverslips were assessed per treatment condition and 100–150 cells were manually counted per treatment. Neuronal survival data is representative of 3 independent experiments, and control levels were not statistically different for normalization purposes. *** p <0.001, vs. vehicle (no insult), ### p <0.001, vs. insult.

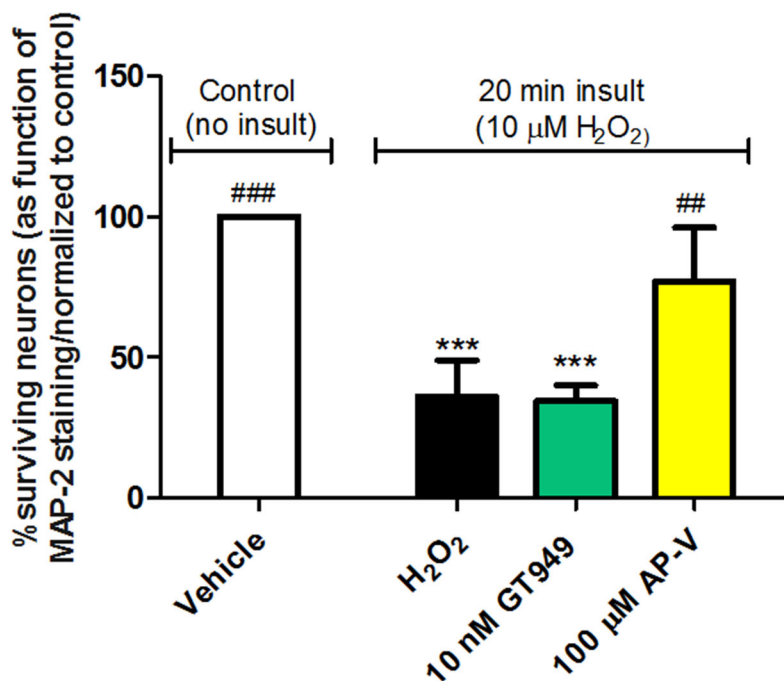


Figure 3. Lack of neuroprotective properties of GT949 in bilaminar cultures after acute exposure to H₂O₂-mediated oxidative stress.

Bilaminar cultures at 14 DIV were treated with conditioned medium containing 10 μM H₂O₂ for 20 minutes, followed by replacement of the H₂O₂ medium and compound treatment for 24 hours. Neurons were then fixed and immunostained against MAP-2 for downstream survival analysis. Exposure significantly reduced neuronal survival, and co-treatment with 10 nM GT949 was not neuroprotective. Co-treatment with 10 nM GT996 also had no effect on neuronal survival after H₂O₂ exposure. However, Co-treatment with 100 μM AP-V did significantly reverse H₂O₂-induced neuronal death, suggesting that NMDA receptor activation is involved in oxidative stress-mediated neuronal toxicity. Survival quantification was normalized to control cultures. 3–4 coverslips were assessed per treatment condition and 100–150 cells were manually counted per treatment. Neuronal survival data is representative of 3 independent experiments and control levels were not statistically different for normalization purposes. ****p<0.0001, vs. control (no insult), ###p<0.001, #p<0.01, vs. insult.

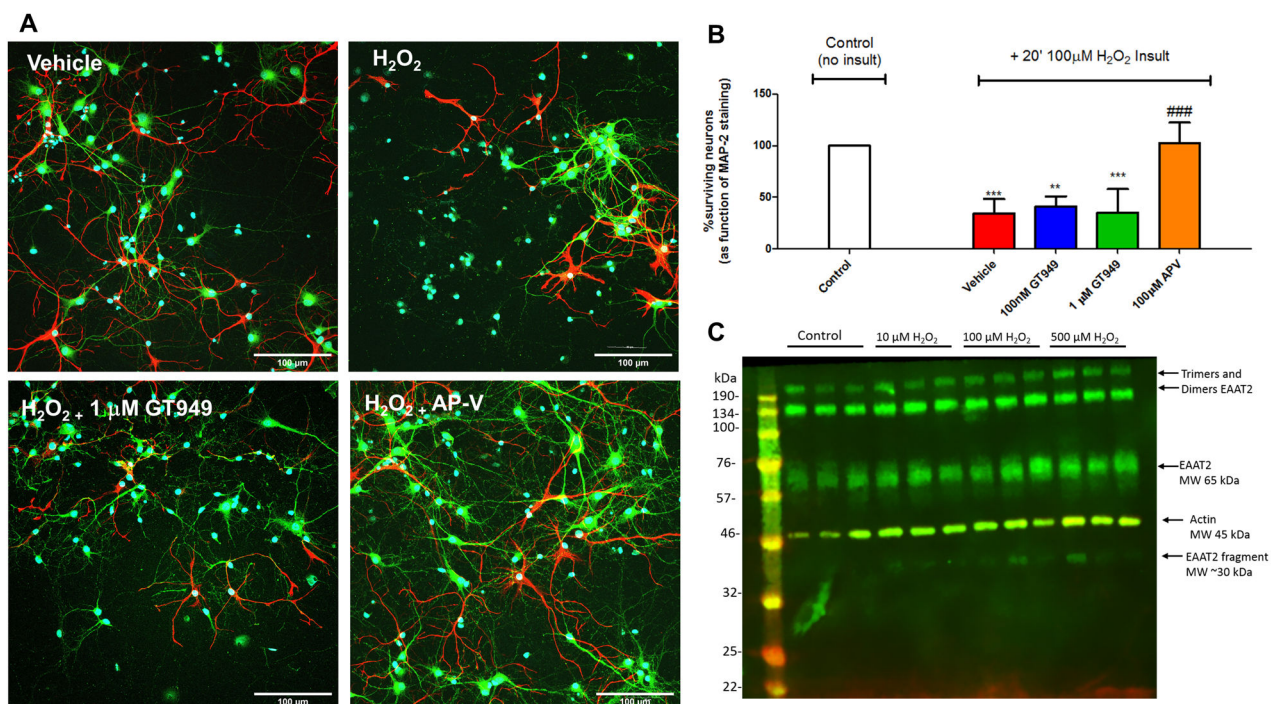


Figure 4. Lack of neuroprotective effects of GT949 in mixed neuron-glia cultures subjected to H₂O₂-mediated oxidative stress.

A. Representative images of cultures after different treatments, with neuronal marker MAP-2 in green, glial marker GFAP in red, and counterstaining with the nuclear marker DAPI (blue). Cultures exposed to vehicle do not display obvious degeneration or cell death. Cultures acutely exposed to H₂O₂ (20 min, 100 μM) show increased DAPI positive, MAP-2 negative cells, indicative of toxicity and neuronal death. Co-exposure of 1 mM GT949 did not change the level of neuronal death, whereas co-incubation with 100 mM AP-V reversed a portion of the cellular damage caused by H₂O₂. All images are shown at 30x magnification. Scale bar: 100 μm.

B. Quantification of neuronal survival. Cultures were treated with 100 μM H₂O₂ for 20 minutes, followed by media replacement and compound treatment for 24 hours. Cultures were then fixed and immunostained for neuronal death analysis as in A. Survival quantification was normalized to control cultures, with 3 coverslips assessed per treatment condition per experiment. Neuronal survival data is representative of 2 independent experiments (n=6) and control levels were not statistically different for normalization purposes. ****p<0.0001, vs. control (no insult), ###p<0.001, ##p<0.01, vs. H₂O₂ insult.

C. Representative immunoblot showing cleavage of the EAAT2 transporter after treatment with higher concentrations of H₂O₂. Each treatment group represents 3 lanes; from left to right: control (no insult), 10 μM, 100 μM and 500 μM H₂O₂. EAAT2 expression is shown in green and beta-actin in red (used as a loading control). MW= Molecular weight.

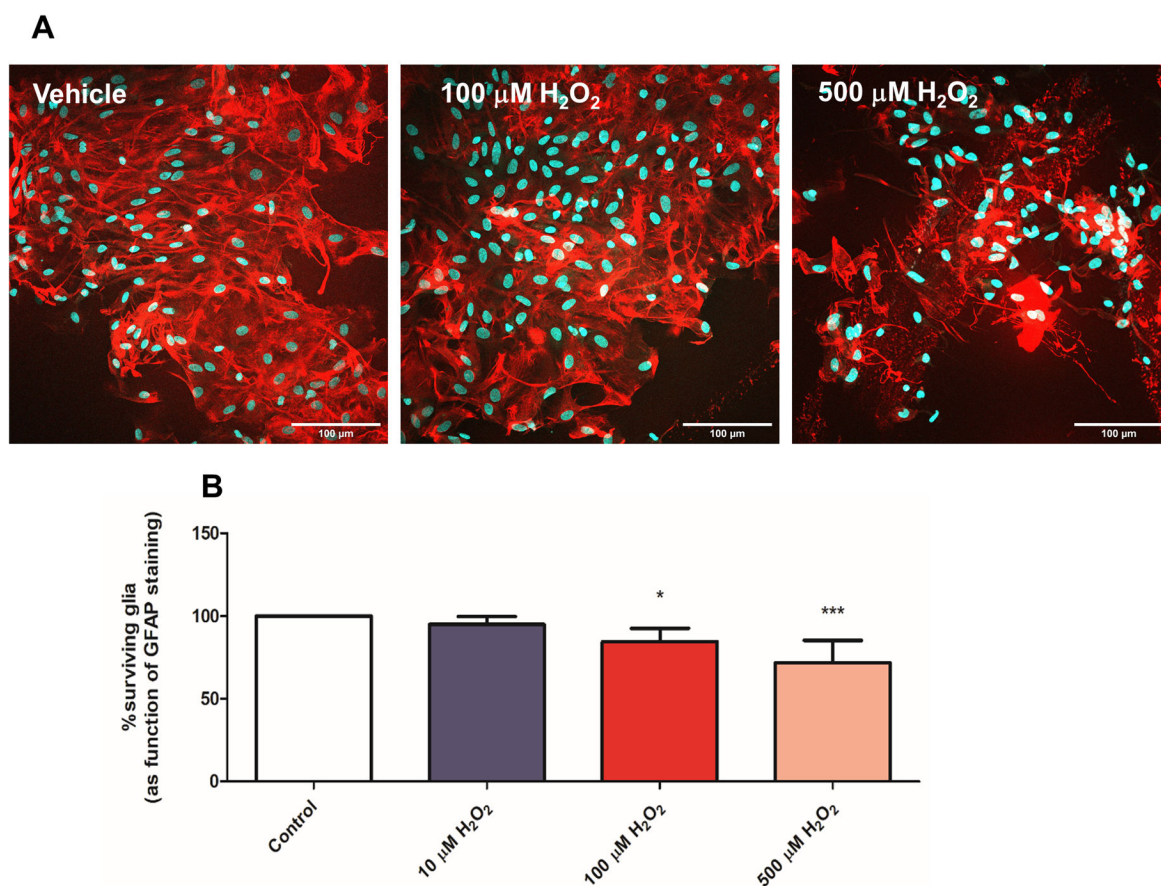


Figure 5. H_2O_2 mediated oxidative stress results in significant glial cell death.

A. Representative images of pure glia cultures incubated with vehicle (control), 100 and 500 μM H_2O_2 for 20 min, indicating loss of glia due to the insults.

B. Quantification of GFAP+ cells indicates 100 μM and 500 μM H_2O_2 insults result in significant glial cell death in pure glial cultures. Survival quantification was determined by comparing GFAP staining to total DAPI staining and cultures were normalized to control, vehicle-treated cultures. 3 coverslips were assessed per treatment condition per experiment. Neuronal survival data is represented as the mean \pm SEM of 2 independent experiments (n=6) and control levels were not statistically different for normalization purposes.

***p<0.001, *p<0.05, vs. control (no insult).

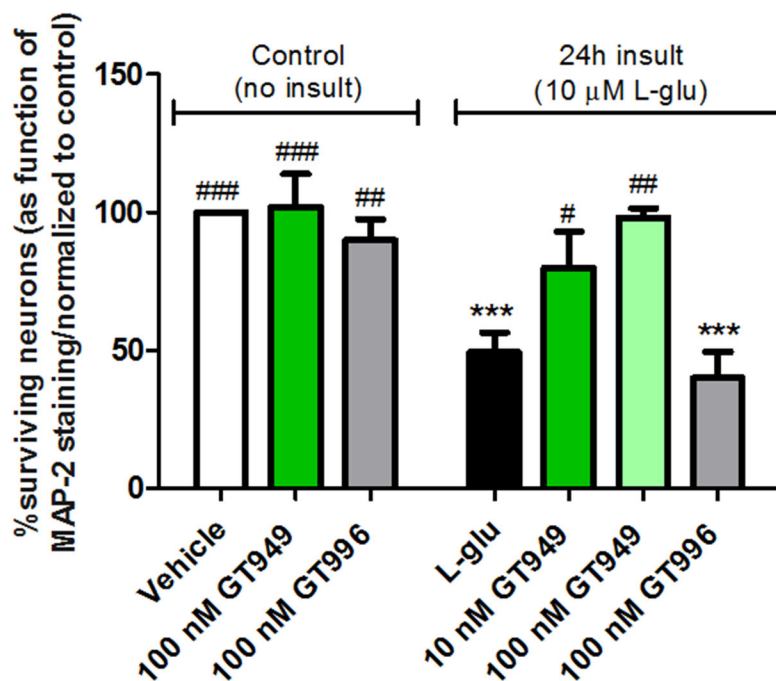


Figure 6. Neuroprotective properties of GT949 in bilaminar cultures after prolonged exposure to glutamate (in the presence of glia).

14 DIV bilaminar cultures were treated with glia-conditioned medium containing 10 μ M L-glutamate and a compound of interest for 24 hours. After the treatment duration, neurons were fixed and immunostained for survival analysis. Again, treatment with 100 nM GT949 or GT996 alone did not alter neuronal survival. As expected, L-glutamate significantly decreased neuronal survival, and GT949 mitigated L-glutamate toxicity. The 100 nM dose of GT949 produced a more significant reversal of cell death than the 10 nM dose, suggesting a dose-response effect of this compound. Furthermore, the inactive GT996 has no effect on L-glutamate toxicity. Data was normalized to control cultures. 3–4 coverslips were assessed per treatment condition and 100–150 cells were manually counted per treatment. Neuronal survival data is representative of 3 independent experiments and control levels were not statistically different for normalization purposes. *** $p < 0.001$, ** $p < 0.01$, vs. control (no insult), ## $p < 0.01$, # $p < 0.05$, vs. insult.

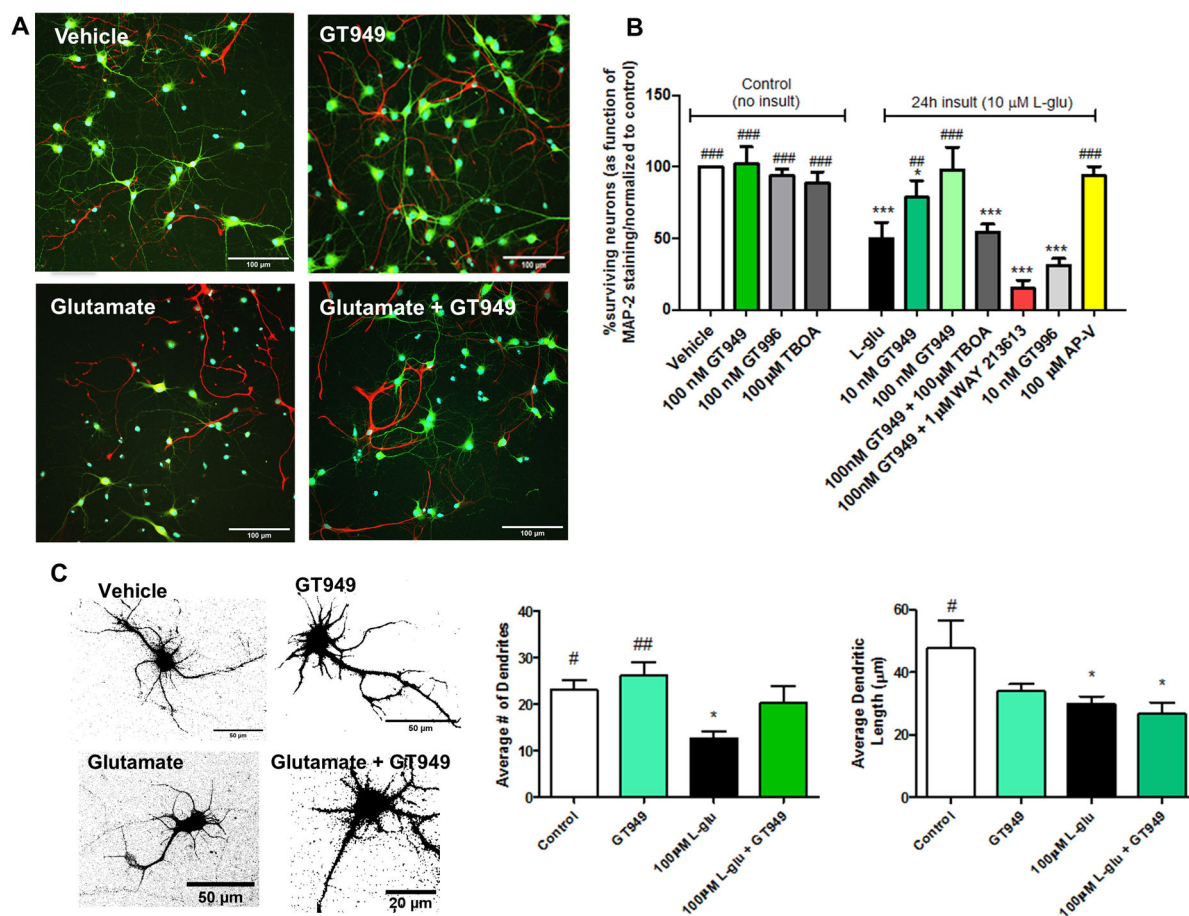


Figure 7. Neuroprotective properties of GT949 in mixed neuron-glia cultures after prolonged exposure to glutamate.

A. Representative images of mixed neuron-glia cultures after treatments with 10 μM L-glutamate, and a compound of interest for 24 hours. Cells were fixed and immunostained for the neuronal marker MAP-2 (green) and the glial marker GFAP (red), and counterstaining with the nuclear marker DAPI (blue). Vehicle and GT949 treated cultures in the absence of L-glutamate do not display obvious degeneration of cell death. Cell death was evident in L-glutamate treated cultures, as assessed by the increased number of DAPI positive, MAP-2 and GFAP negative cells. Co-treatment with 100 nM GT949 reversed a portion of the L-glutamate toxicity by increasing MAP-2 and GFAP expression and reducing the number of DAPI-only cells. All images are shown at 40x magnification. Scale bar: 50 μm.

B: Quantification of neuronal survival in mixed cultures (normalized to control, vehicle-treated cultures). Treatment with 100 nM GT949, GT996, or 100 μM TBOA (glutamate transporter inhibitor) alone did not affect neuronal survival levels. L-glutamate significantly reduced neuronal survival, which was mitigated by co-treatment with GT949 and the NMDA antagonist AP-V. The 100 nM concentration of GT949 produced a more significant neuroprotective effect than the 10 nM concentration. Co-treatment with GT949 and the glutamate transporter inhibitor TBOA and the selective EAAT2 inhibitor WAY 23 213613 did not rescue neuronal damage in L-glutamate exposed cultures, suggesting that GT949 neuroprotection requires active glutamate transporters, specifically EAAT2. Co-treatment

with inactive analog GT996 did not alter glutamate toxicity. 3–4 coverslips were assessed per treatment condition, and 100–150 cells were manually counted per treatment. Neuronal survival data is representative of 3 independent experiments and control levels were not statistically different for normalization purposes. *** $p < 0.001$, * $p < 0.05$, vs. control (no insult), ### $p < 0.001$, ## $p < 0.01$, vs. insult.

C. Representative images of neurons from 14 DIV mixed neuron-glia cultures under control conditions, incubated with 100 nM GT949 for 24 hours without insult, under 100 μ M L-glutamate insult conditions for 24 hours, and with 100 μ M L-glutamate co-applied with 100 nM GT949. Scale bar = 50 μ M. Sholl analysis on the right shows that glutamate insult resulted in decreased dendritic number and average dendritic length. Co-application with GT949 results in a trend in increasing the total number of dendrites, although it did not have effects on average length of dendrites. * $p < 0.05$, vs. control (no insult), ## $p < 0.01$, # $p < 0.05$, vs. glutamate insult.

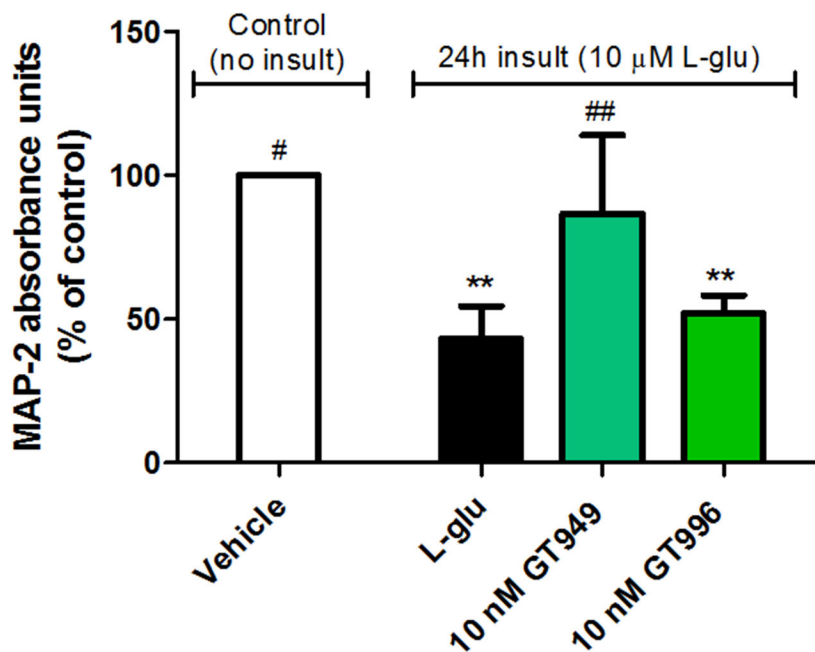


Figure 8. MAP-2 ELISA study of neuroprotective properties of GT949 in mixed neuron-glia cultures after prolonged exposure to glutamate.

Mixed cultures were treated with 10 μ M L-glutamate, and a compound of interest for 24 hours, followed by MAP-2 ELISA. L-glutamate exposure significantly reduced expression of MAP-2, as expected from previous experiments analyzing neuronal survival by MAP-2 immunostaining. Additionally, GT949 reversed the MAP-2 deficit in L-Glutamate exposed cultures, while the inactive analog GT996 had no effect. Data from at least 12 wells per treatment was plotted as a function of absorbance, which correlates to the amount of MAP-2 binding. Data was obtained from 3 independent experiments, and normalized to control values. ** $p < 0.01$, vs. control (no insult), ## $p < 0.01$, # $p < 0.05$, vs. insult.