Activation of group I metabotropic glutamate receptors reduces neuronal apoptosis but increases necrotic cell death *in vitro*

JW Allen[1,2], SM Knoblach[1,3] and AI Faden*[,1,3,4]

[1] Institute for Cognitive and Computational Sciences, Georgetown University Medical Center, Washington, DC 20007, USA

[2] Interdisciplinary Program in Neuroscience, Georgetown University Medical Center, Washington, DC 20007, USA

[3] Department of Neuroscience, Georgetown University Medical Center, Washington, DC 20007, USA

[4] Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007, USA

* Corresponding author: AI Faden, EP-04 Research Building, 3970 Reservoir Road, N.W. Washington, DC 20007, USA. Tel: (202) 687 0492; Fax: (202) 687 0617; E-mail: fadena@giccs.georgetown.edu

Abstract

Glutamate released during acute CNS insults acts at metabotropic glutamate receptors (mGluR), including group I mGluR. Blockade of group I mGluR during *in vitro* neuronal trauma provides neuroprotection, whereas activation exacerbates such injury. However, the effects of group I mGluR agonists or antagonists have been primarily studied in *in vitro* models characterized by necrotic cell death. We examined the role of group I mGluR in the modulation of neuronal injury induced during oxygen-glucose deprivation (OGD), a well-studied model of necrosis, and by application of two well established pro-apoptotic agents: staurosporine and etoposide. Inhibition of group I mGluR attenuated necrosis induced by OGD, whereas selective activation of group I mGluR exacerbated such injury. In contrast, activation of group I mGluR, including selective activation of mGluR5, significantly attenuated apoptotic cell death induced by both staurosporine and etoposide. This effect was completely reversed by co-application of a group I mGluR antagonist. Thus, group I mGluR appear to exhibit opposite effects on necrotic and apoptotic neuronal cell death. Our findings suggest that activation of mGluR1 exacerbates neuronal necrosis whereas both mGluR1 and mGluR5 play a role in attenuation of neuronal apoptosis. *Cell Death and Differentiation* (2000) **7**, 470–476.

Abbreviations: 4CPG, (S)-4-carboxyphenylglycine; AIDA, (R,S)-1-aminoindan-1, 5-dicarboxylic acid; CHPG, (R,S)-2-chloro-5-hydroxyphenylglycine; DHPG, dihydroxyphenylglycine; MK801, dizocilpine; LDH, lactate dehydrogenase; MCPG, -methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptors; OGD, oxygen-glucose deprivation; TBI, traumatic brain injury

Introduction

Activation of both ionotropic and metabotropic glutamate receptors has been implicated in the pathophysiology of stroke and CNS trauma. It has been well established that blockade of ionotropic glutamate receptors provides neuroprotection *in vitro* and *in vivo*.[1−5] Recent studies have suggested that metabotropic glutamate receptors (mGluR) are also activated following traumatic brain injury (TBI)[6−9] or cerebral ischemia,[10] or *in vitro* neuronal trauma[7,9] or ischemia,[11,12] leading to neuroprotection or exacerbation depending upon the mGluR subtype activated.

MGluR have been divided into three groups, based on sequence homology and signal transduction mechanisms. Group I receptors (mGluR1, mGluR5) stimulate phospholipase C, leading to phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization; group II and group III receptors are negatively coupled to adenylyl cyclase activity, but differ in their pharmacological properties.[13]

Selective activation of group I mGluR potentiates NMDA-induced neuronal death in cultured neurons,[12,14] and exacerbates *in vitro* trauma[5,7] and oxygen-glucose deprivation (OGD).[11,12] Inhibition of group I mGluR provides neuroprotection against *in vitro* trauma,[5,7] and may protect against OGD.[12] Moreover, group I mGluR blockade is also protective against CNS insults *in vivo*.[6,7,10]

Few studies have examined the effects of group I mGluR modulation on apoptotic cell death. Copani *et al.* reported that activation of group I mGluR protects against apoptosis of cerebellar granule cells grown in medium containing low levels of potassium,[15,16] or cerebellar granule cells incubated with β-amyloid.[17] However, the latter study used either non-selective agents or compounds with actions at multiple mGluR subtypes. More recently, activation of group I mGluR has been demonstrated to protect against apoptotic cell death and to decrease caspase-3 activity induced by nitric oxide treatment.[18,19]

Because of the importance of glutamate in the pathogenesis of acute CNS injury and the fact that both apoptotic and necrotic cell death occur after such insults, we examined the effects of selective group I mGluR activation or inhibition in models demonstrating either predominantly necrotic or apoptotic cell death. OGD of rat cortical neuronal-glial cultures was used as a model of neuronal necrosis.[12] Chronic application of staurosporine

or etoposide to rat cortical neuronal-glial cultures was used to induce selective neuronal apoptosis.[20]

Results

Effects of group I mGluR on necrotic injury

Rat neuronal-glial cultures (17–19 DIV) were subjected to brief (30–110 min) OGD followed by oxygen and glucose replacement. Cell death was estimated by LDH release assay 24 h after injury. Increasing length of exposure to OGD conditions produced a progressive rise in neuronal injury, with 60 min of OGD inducing 50% neuronal death and 90 min of OGD producing nearly complete neuronal death (Figure 1). Consistent with a glutamate-mediated necrotic injury, pretreatment of neuronal-glial cultures with MK801 (10 μM) produced nearly complete neuroprotection at 60, 75, and 90 min OGD. After 90 min of OGD, detectable neuronal injury occurred even in the presence of MK801 (Figure 1).

Using 60 min of OGD as a model of necrotic neuronal death,[21] we investigated the effect of selective group I mGluR activation or inhibition. Administration of the group I mGluR antagonists MCPG (500 μM), AIDA (200 μM), or 4CPG (30 μM) elicited significant neuroprotection against OGD-induced LDH release at 24 h post-injury (Figure 2A). The effects of the individual group I mGluR antagonists were not statistically different from one another. Application of AIDA (200 μM) immediately following 60 min OGD also provided significant neuroprotection (AIDA 66 ± 14 versus OGD 100 ± 13, n=31, $P < 0.05$, Student's t-test). In contrast to the neuroprotective effects of group I mGluR inhibition, the selective group I agonist DHPG (20 μM) induced

significant exacerbation of cell death after OGD (Figure 2B).

Effects of group I mGluR on apoptosis induced by etoposide and staurosporine

Treatment of neuronal-glial cultures with staurosporine, a nonselective protein kinase inhibitor, for 24 h at a concentration of 0.3 μM, which produces neuronal apoptosis with little glial damage,[20] induces a qualitative increase in the number of cells exhibiting apoptotic-like nuclear morphology after staining with Hoechst 33258 (Figure 3). Quantitatively, staurosporine induces approximately $73 \pm 6\%$ neuronal cell death at 24 h as determined by LDH release following total neuronal death in sister neuronal-glial cultures treated with 200 μM NMDA for 24 h (n=16). Qualitative increases in the
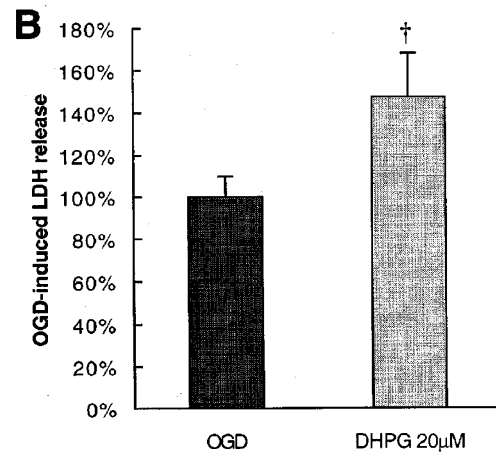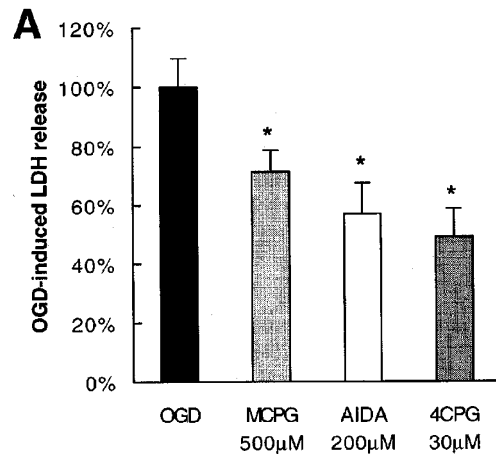
Figure 2 Inhibition of group I mGluR protects against OGD, whereas activation of group I mGluR exacerbates this injury. (A) Pre-and post-treatment with the group I/II mGluR antagonist MCPG (light gray bar), selective group I mGluR antagonist AIDA (white bar), or group I mGluR antagonist and group II mGluR agonist 4CPG (dark gray bar) significantly decreased LDH release at 24 h induced by 60 min OGD (black bar). (B) Selective pre- and post-injury activation of group I mGluR by DHPG (dark gray bar) significantly exacerbated 60 min OGD (black bar). Bars represent mean $\pm$ S.E.M., n=30–32 cultures per condition. Data expressed as a percentage of OGD-induced LDH release. *$P < 0.05$ vs OGD (ANOVA followed by Student-Newman-Keuls test); †$P < 0.05$ vs OGD (Student's t-test)
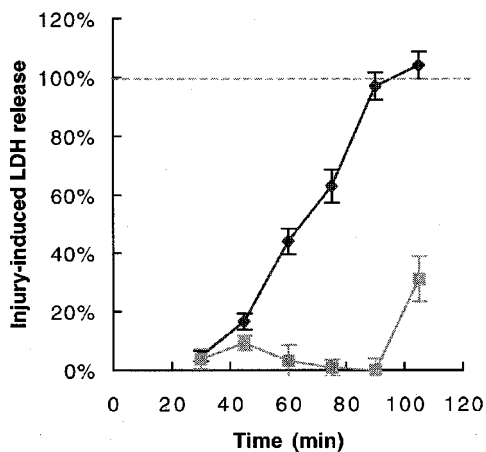
Figure 1 Oxygen-glucose deprivation (OGD) induces progressive neuronal death. Rat neuronal-glial cultures were subjected to increasing duration of OGD and cell death was estimated after 24 h by measurement of LDH released into the media as described in Materials and Methods. Complete neuronal death in control cultures was determined by application of 200 μM NMDA for 24 h (dotted line). Complete neuronal death was apparent after 90 min exposure to OGD conditions in the absence of treatment (black diamonds). MK801 produced nearly complete attenuation of cell death induced by up to 90 min OGD conditions (gray squares). After 90 min of exposure to OGD conditions, cell death was apparent even in those cultures treated with MK801. Values represent mean $\pm$ S.E.M., n=32 cultures per condition. Data expressed as a percentage of NMDA-induced LDH release
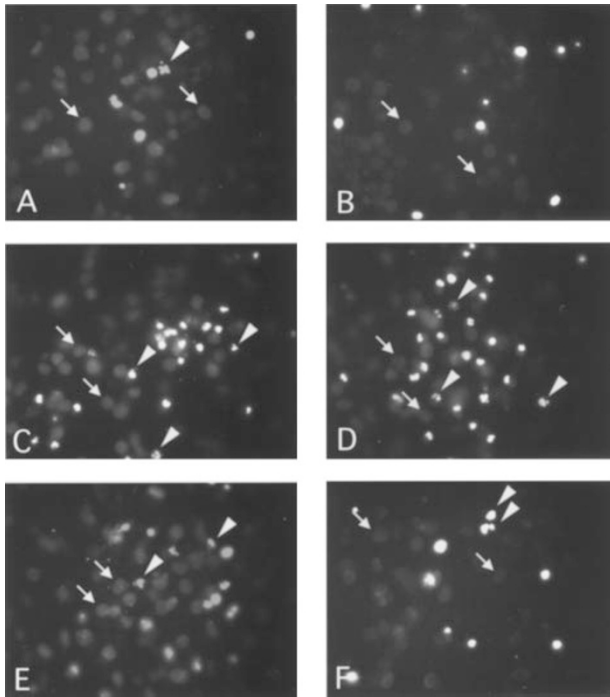
Figure 3 Staurosporine and etoposide induce nuclear condensation and fragmentation consistent with apoptotic cell death. A small number of cells exhibited condensed or fragmented in nuclei neuronal-glial cultures 24 h after addition of vehicle (**A,B**). A qualitative increase in the number of cells displaying condensed or fragmented nuclei was observed after 24 h incubation with staurosporine (0.3 μM) (**C**) or etoposide (2.5 μM) (**D**) compared with vehicle controls. A qualitative decrease in the number of the condensed or fragmented nuclei was seen after the application of DHPG (20 μM) to neuronal-glial cultures treated with staurosporine (**E**) or etoposide (**F**). Viable neurons (arrows) exhibit diffuse nuclear staining whereas apoptotic neurons (arrowheads) are characterized by condensed and fragmented nuclei after staining with Hoechst 33258. Images were obtained at 600 $\times$ after staining with Hoechst 33258 to visualize nuclear morphology as detailed in Materials and Methods

number of cells exhibiting nuclear condensation or fragmentation occurs after treatment for 24 h with 2.5 μM etoposide, an inhibitor of topoisomerase II (Figure 3). Similar to staurosporine, this concentration of etoposide induces 77 $\pm$ 3% neuronal death by 24 h (*n*=16–21).

Staurosporine and etoposide treatment were used as models of apoptotic neuronal injury in which to study the effects of selective group I mGluR activation or inhibition. Incubation of neuronal-glial cultures with DHPG (20–100 μM) significantly attenuated both staurosporine- and etoposide-induced increases in LDH release (Figure 4). Apoptosis induced by staurosporine or etoposide was insensitive to AIDA (200 μM) treatment (Figure 4). However, co-administration of AIDA with DHPG completely reversed the neuroprotective effects of DHPG (Figure 4). A qualitative decrease in the number of cells with apoptotic-like morphology was also observed after treatment with DHPG in the presence of staurosporine or etoposide (Figure 3). This decrease was confirmed by quantitative counts of the number of cells containing fragmented or condensed nuclei following Hoechst 33258 (Figure 5).

The neuroprotective effects of group I mGluR activation were confirmed using a second agonist, CHPG, that is
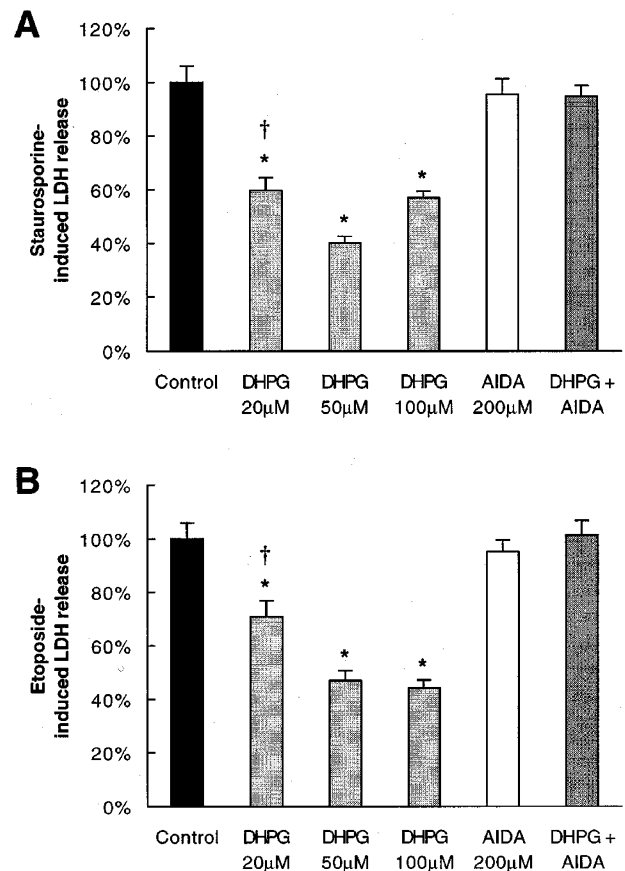

Figure 4 Activation of group I mGluR protects against staurosporine- and etoposide-induced cell death. (**A**) Application of the group I mGluR agonist DHPG at three doses significantly attenuated LDH release at 24 h induced by staurosporine (0.3 μM). In contrast, the group I mGluR antagonist AIDA was without effect on this injury but co-application of AIDA (200 μM) with DHPG (20 μM) completely reversed the protective effects of group I mGluR activation. (**B**) Administration of DHPG (20–100 μM) also significantly protected against cell death at 24 h induced by etoposide (2.5 μM). Analogous to the lack of effect of AIDA in staurosporine-induced injury, AIDA had no effect on this injury, but completely reversed the protective effects of DHPG (20 μM) when administered simultaneously. Combined treatment with a group I mGluR agonist and antagonist was performed using 20 μM DHPG and 200 μM AIDA. Bars represent mean $\pm$ S.E.M., *n*=23–53 cultures per condition. Data expressed as a percentage of staurosporine- (**A**) or etoposide-induced (**B**) LDH release. *$P < 0.05$ *vs* staurosporine (**A**) or etoposide (**B**) treatment; †$P < 0.05$ *vs* AIDA+DHPG (ANOVA followed by Student-Newman-Keuls test)

reported to be specific for the group I mGluR mGluR5 subtype.[22] Protection against staurosporine- and etoposide-induced increases in LDH release was observed after administration of 20 μM CHPG (Figure 6).

Discussion

Our data support the hypothesis that the effects of group I mGluR activation during neuronal injury are dependent upon the type of cell death. Inhibition of group I mGluR with a variety of antagonists attenuates necrosis induced by OGD, whereas selective activation of group I mGluR by the group I agonist DHPG exacerbates such injury. In contrast, DHPG significantly attenuates apoptotic cell death induced by both
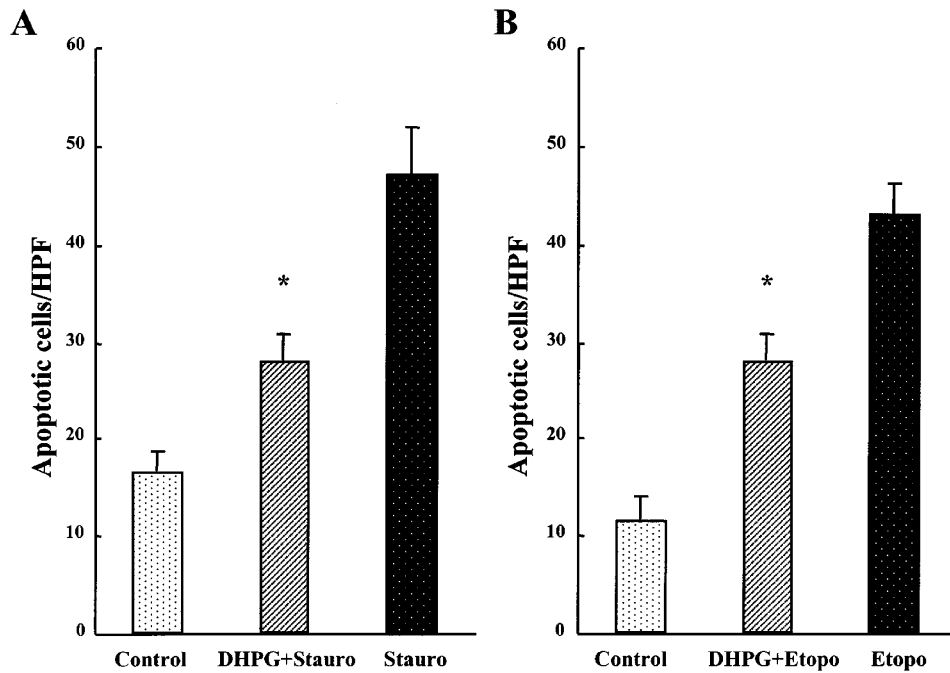
Figure 5 Group I mGluR activation elicits significant reduction in the number of apoptotic cells following staurosporine or etoposide treatment. (**A**) Staurosporine-induced increases in cells containing condensed or fragmented nuclei stained by Hoechst 33258 is significantly attenuated by application of DHPG (50 μM). (**B**) Similarly, DHPG (50 μM) also reduces the number of apoptotic cells counted after etoposide treatment. Cell counts were performed at 400 $\times$ by selecting a random field as detailed in Materials and Methods. Bars represent mean $\pm$ S.E.M., n=5 – 11 cultures per condition. *P<0.05 *vs* staurosporine (**A**) or etoposide (**B**) treatment (ANOVA followed by Student-Newman-Keuls test). HPF=high power field
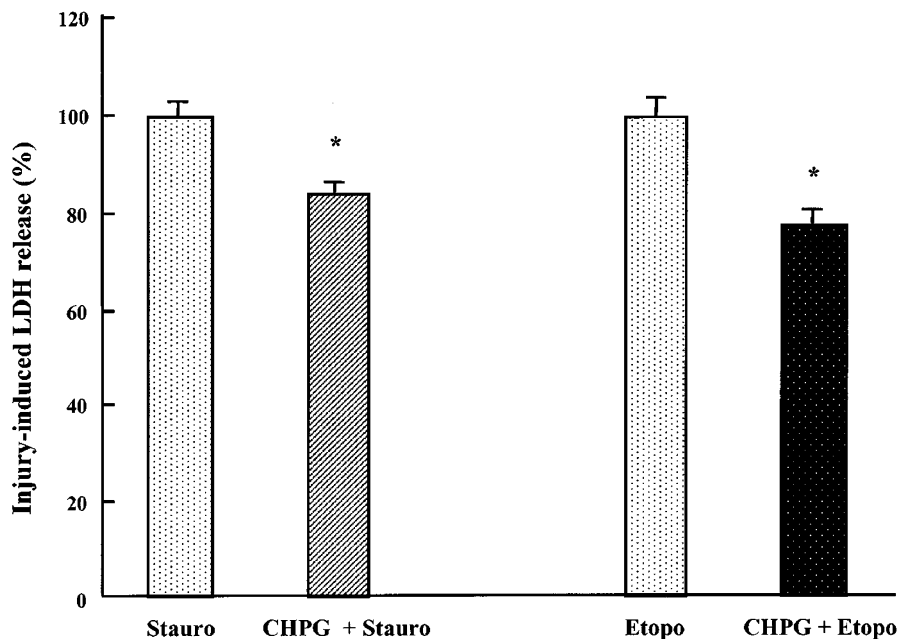

Figure 6 Selective activation of mGluR5 protects against staurosporine- or etoposide-induced cell death. Application of CHPG (20 μM) during treatment with staurosporine (Stauro) or etoposide (Etopo) significantly reduces LDH release at 24 h. Bars represent mean $\pm$ S.E.M., n=21 – 24 cultures per condition. Data expressed as a percentage of staurosporine- or etoposide-induced LDH release. *P<0.001 *vs* staurosporine or etoposide treatment (Student's t-test)

staurosporine and etoposide. This effect is completely reversed by co-application of the group I mGluR antagonist AIDA.

The pharmacologic profiles of the agents used to modulate group I mGluR in this study have been previously reported. Three group I mGluR antagonists

were tested in OGD-mediated injury. MCPG is an antagonist at both group I and II mGluR.[23] 4CPG is an antagonist at group I mGluR and an agonist at group II mGluR.[23] AIDA is a selective antagonist at group I mGluR,[24] with more activity at mGluR1 than mGluR5.[25] DHPG has been reported to be a selective agonist of group I mGluR, with little or no effect on the other mGluR subtypes or ionotropic glutamate receptors studied.[26,27] CHPG selectively activates the mGluR5 subtype of group I mGluR with no reported activity at mGluR1.[22]

A previous study using non-selective group I mGluR agonists suggested that activation of group I mGluR exacerbates OGD.[12] However, the selective effects of group I mGluR inhibition *in vitro* have not been reported. Consistent with the work of Choi and colleagues,[12] activation of group I mGluR by DHPG exacerbates OGD in our cultures. Moreover, similar to the effects on traumatic injury *in vitro*,[28] AIDA-mediated group I mGluR inhibition significantly protected against cell death induced by OGD.

Modulation of group I mGluR may affect necrotic injury through a variety of mechanisms. Although activation of group I mGluR has been primarily associated with activation of phospholipase C, leading to phosphoinositide hydrolysis and mobilization of intracellular calcium by inositol triphosphate and activation of protein kinase C (PKC) by diacylglycerol, group I mGluR activation has also been associated with increases in cyclic AMP, liberation of arachidonic acid, potentiation of NMDA receptors, and complex modulation of potassium and calcium channels.[13] We have previously demonstrated that whereas antisense directed against mGluR1 or mGluR5 equally reduced group I mGluR-mediated increases in inositol phosphate accumulation, only antisense against mGluR1 significantly attenuated traumatic neuronal injury *in vitro*, a model of neuronal necrotic cell death.[7] Therefore, increases in inositol triphosphate with resulting mobilization of intracellular calcium may not mediate group I mGluR-induced exacerbation of necrotic injury. In addition, we have previously shown in the same model that group I mGluR-mediated exacerbation occurs in the presence of NMDA receptor blockade, suggesting that potentiation of NMDA receptors is not the sole mechanism whereby group I mGluR worsen necrotic cell death.[28] It remains to be determined whether activation of PKC, increases in cAMP, liberation of arachidonic acid, or modulation of calcium or potassium channels play a role in group I mGluR-mediated effects on necrotic injury.

In contrast to the exacerbating effect of group I mGluR activation on necrotic cell death, DHPG protected against well-characterized models of apoptosis induced by staurosporine and etoposide. This effect appears to be due to direct activation of group I mGluR, as co-application of AIDA completely reversed DHPG-mediated neuroprotection. Treatment with AIDA alone had no effect on either staurosporine- or etoposide-induced cell death. This is unlikely to be secondary to insufficient inhibition of group I mGluR as the doses of DHPG and AIDA used in all experiments were at least twice the reported EC_{50} values for these compounds.[13,24] Such concentrations were also shown to be maximally effective in mechanical traumatic

injury using similar neuronal-glial cultures.[7,28] Furthermore, this concentration of AIDA significantly exacerbates neuronal apoptosis induced by β-amyloid.[29]

While activation of group I mGluR may exacerbate necrotic cell death through modulation of the mGluR1 subtype as discussed above, neuroprotection produced by group I mGluR activation in the setting of apoptotic injuries is at least in part mediated via mGluR5. CHPG is a selective agonist at mGluR5 subtype with no reported agonist action at mGluR1.[22] Similar to DHPG, this compound protects against both staurosporine and etoposide. Therefore, such protection against apoptosis may be mediated by mGluR5 activation. However, as AIDA completely reversed the neuroprotective effects of DHPG, and this compound is more selective for mGluR1 than for mGluR5, it is likely that both group I mGluR subtypes participate in the modulation of apoptosis.

The protective effects of DHPG against staurosporine- or etoposide-induced apoptosis may result from the ability of group I mGluR agonists to increase intracellular calcium levels.[30,31] Although staurosporine does not alter intracellular calcium levels, raising intracellular calcium protects against staurosporine-induced neuronal apoptosis.[20] Moreover, decreasing intracellular calcium levels potentiates apoptosis in other *in vitro* systems.[32,33] Therefore, group I mGluR activation may protect against apoptosis by increasing release of calcium from intracellular stores or through potentiation of calcium channel activation.[34]

The opposite effects of group I mGluR modulation on necrosis and apoptosis presented here may have relevance for the possible therapeutic potential of group I mGluR antagonists in the treatment of CNS injury. Further studies using *in vivo* models of apoptosis and necrosis are warranted to determine if the differential effects of group I mGluR are also present *in vivo*. If inhibition of group I mGluR increases apoptotic cell death after *in vivo* injury, these findings suggest that the effectiveness of group I mGluR antagonists in CNS trauma and ischemia may be substantially enhanced by simultaneously blocking apoptosis.

Materials and Methods

Tissue culture

Glia were prepared from 1–3-day-old Sprague-Dawley rat cortices (Taconic Farms, Germantown, NY, USA) and neurons were prepared from 18-day-old Sprague-Dawley rat embryonic cortices as previously described in detail.[35] Briefly, dissociated cortices from 1–3-day-old rats were seeded in 96-well Primaria microplates (Falcon, Lincoln Park, NJ, USA) and were allowed to grow to confluency. Isolated embryonic cortices were dissociated and individual cells ($2–2.5 \times 10^6$ cells/ml) were seeded on 10-day-old *in vitro* (DIV) confluent glial cultures. Cortices were dissociated in Hank's balanced salt solution without calcium or magnesium (Mediatech, Herndon, VA, USA) supplemented with 10 mM HEPES (pH 7.0; Biofluids, Rockville, MD, USA) and 1 mM sodium pyruvate (Biofluids). Cultures were fed twice per week by replacement of one-third of media with minimal essential medium with Earle's salts (Mediatech) supplemented with 10% equine serum (HyClone Laboratories, Logan, UT, USA), 27.5 mM HEPES (pH

7.2), 2 mM glutamine (Biofluids), 20 mM glucose (Biofluids), and 1% antibiotic-antimycotic solution (Biofluids). Ara-C (10 μM; Sigma, St. Louis, MO, USA) was added during the first feeding to stop further glial proliferation. After 10 DIV, glutamine concentration was reduced to 1 mM and equine serum was omitted. Cultures were incubated at 37°C in humid atmosphere with 4% CO_2. Neuronal-glial cultures were used at 19–21 DIV.

Induction of oxygen-glucose deprivation (OGD) injury

Neuronal-glial cultures 17–20 DIV were used for OGD experiments. Atmosphere was evacuated from Anaerobic System Model 1025 (Forma Scientific, Marietta, OH, USA) and replaced with 5% CO_2, 10% H_2, and 85% N_2. Remaining O_2 was scavenged by a palladium catalyst (Forma Scientific). Deoxygenated balanced salt solution (BSS; 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 0.01 mM glycine, and 10 mg/L phenol red) was prepared by bubbling with the anaerobic gas mixture for 15 min prior to starting experiments. Oxygen levels were less than 0.3% in treated BSS as measured by a dissolved oxygen meter (Orion Model 830, Boston, MA, USA). Neuronal-glial cultures in 96-well microplates were transferred into the anaerobic chamber through an air lock and then washed seven times using Skan Washer 300 automated plate washer with deoxygenated BSS (greater than $2000 \times$ dilution). All drugs were resuspended in the smallest amount of solvent possible and then diluted with deoxygenated BSS in the chamber. Control cultures were treated with vehicle diluted in the same manner to control for residual oxygen in the solvent. After adding compounds to appropriate wells, cultures were placed in an incubator at 37°C within the chamber. After 30–105 min, cultures were removed from the incubator and washed seven times with deoxygenated BSS and removed from the chamber. Reperfusion was performed by immediately adding an equal volume of oxygenated BSS supplemented with 5.5 mM glucose, 1% antibiotic-antimycotic and vehicle or drug where appropriate. Cultures were then placed at 37°C for 24 h before cell death assessment by LDH release assay.

Staurosporine and etoposide treatment

Neuronal-glial cultures 17–20 DIV were treated with staurosporine or etoposide to induce apoptosis. Staurosporine (Sigma) was diluted to 214 μM with dimethyl sulfoxide (DMSO; Sigma) and stored at 4°C. Etoposide (Sigma) was diluted to 500 μM with DMSO and stored at -20°C. Cultures were washed with BSS using an automated plate washer. An equal volume of BSS supplemented with 5.5 mM glucose, 1% antibiotic-antimycotic, staurosporine or etoposide, and drug or vehicle was added to each well. Cultures were then returned to 37°C for 24 h. After removal of two-thirds of the media for LDH release assay, some cultures were stained with Hoechst 33258 as described below.

Cell death assessment

Total cell death was estimated using LDH release, which has been widely used as a biochemical measure of cell injury.[36–38] Briefly, 24 h after trauma, 75 μl media was transferred to a 96-well microplate and diluted with 150 μl LDH assay reagent containing 5 mM β-NAD (Sigma), 25 mM lactic acid (Sigma), 0.03% bovine serum albumin (BSA; Sigma), 100 mM Trizma (Sigma) and 0.9% NaCl (pH 8.45; Sigma). Spectrophotometric analysis was performed at room temperature using a Ceres 900 microplate reader (Biotek Instruments, Inc., Winooski, VT, USA) measuring optical density at 340 nm over 250 s at 5 s intervals (50 readings per sample). Linear regression analysis provided an estimate of LDH activity. Basal LDH activity levels were subtracted from treatments prior to analysis.

Hoechst 33258 staining

Hoechst 33258 (Sigma) was diluted in deionized water to 10 mg/ml and stored at -20°C. Hoechst 33258 was added to culture media to a final concentration of 2 μg/ml and incubated at 37°C for 10 min. Cultures were then washed with BSS and examined using a Nikon TE300 microscope (Nikon, Melville, NY, USA) with excitation at 360 nm and emission at 460 nm. Images were captured using an Optronics DEI-750 digital camera (Optronics, Goleta, CA, USA) and Scion Image 1.62a software.

Cell counts

Counts of cells exhibiting nuclear morphology consistent with apoptosis was performed as previously described.[39] Briefly, cultures were stained using Hoechst 33258 after treatment with staurosporine or etoposide in the presence or absence of DHPG as detailed above. Counts were performed at $400 \times$ using a Nikon TE300 microscope with excitation at 360 nm and emission at 460 nm. A random field was selected for each culture and the number of cells containing condensed or fragmented nuclei were counted.

Drugs

(R,S)-1-aminoindan-1, 5-dicarboxylic acid (AIDA), (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG), (S)-4-carboxyphenylglycine (4CPG), dizocilpine (MK801), dihydroxyphenylglycine (DHPG), and -methyl-4-carboxyphenylglycine (MCPG) were all purchased from Tocris Cookson.

Acknowledgments

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