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Neuronal and glial mGluR5 modulation prevents stretch-induced enhancement of NMDA receptor current

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Abstract

Neuronal stretching in culture has been used to model diffuse axonal injury caused by head trauma, and activation of *N*-methyl-D-aspartate receptors (NMDARs) has been implicated in the pathophysiology of such injury. Here we report the effects of modulating injury severity and the metabotropic glutamate receptor subtype 5 (mGluR5) on NMDAR activity after stretch injury. Following mild stretch, cortical neurons plated upon a confluent layer of astrocytes (NG) exhibited both increased maximal current (I_{NMDA}) and reduction in the voltage-dependent Mg^{2+} block. In contrast, neurons grown without an astrocyte monolayer (PN) only exhibited increased I_{NMDA}. In NG, surprisingly, pretreatment with either the mGluR5 agonist CHPG or the mGluR5 antagonist MPEP decreased the enhancement of I_{NMDA}. In contrast, in PN, MPEP similarly limited I_{NMDA} changes, but CHPG was without effect. In both culture conditions, MPEP, but not CHPG, limited the stretch-reduced Mg^{2+} block. Severe stretch had no effect on I_{NMDA} or the Mg^{2+} block in either culture condition, despite a correlation between injury severity and the release of lactose dehydrogenase measured postinjury. Neither CHPG nor MPEP had any direct effects upon the NMDA receptor. We conclude that mGluR5 regulates NMDAR activity during mild stretch injury, but not severe injury, by modulating both the Mg^{2+} block and I_{NMDA}.

Keywords: Glutamate receptor; Excitotoxicity; Stretch injury; Head trauma; Cortical neurons; Neuronal–glial interaction

1. Introduction

Glutamate regulates CNS function through two major classes of receptors: the ionotropic (iGluR) and the metabotropic (mGluR) glutamate receptors. Most fast excitatory synaptic transmissions occur via the iGluRs, whereas slower second messenger-mediated affects occur following activation of the mGluRs (for reviews, see Anwyl, 1999; Bleakman and Lodge, 1998; Dingledine et al., 1999). The iGluRs, categorized by the pharmacological agonists to which they bind, include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA) and *N*-methyl-D-aspartate receptors (NMDARs). The iGluRs are ligand-gated ion channels, permeable to potassium (K^+), sodium (Na^+), and, in the case of NMDA receptors and some AMPA receptors, calcium (Ca^{2+}) ions. In contrast, the mGluRs are G-protein-coupled receptors that either utilize inositol triphosphate, diacylglycerol, or cAMP to mediate their actions, which include regulation of ion channels or modulation of glutamate receptors, among others (for a review, see Anwyl, 1999). The mGluRs are categorized into three groups based upon sequence, pharmacology, and function (for reviews, see Anwyl, 1999; Conn and Pin, 1997; Schoepp et al., 1999). Group I mGluRs (mGluR1 and 5) activate phospholipase C via G_q proteins and initiate an inositol triphosphate/diacylglycerol (IP_3/DAG) second messenger cascade, whereas Group II mGluRs (mGluR2 and 3) and the Group III mGluRs (mGluR4, 6, 7, and 8) inhibit adenylylcyclase via $G_{i/o}$ proteins.

Multiple in vivo and in vitro studies support a role for iGluR and mGluR in cell death following traumatic neuronal injury (for a review, see Temple et al., 2001). Whereas inhibition of iGluRs provides protection against both cell

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injury and neurological dysfunction (Faden et al., 1989; Gill, 1994; McIntosh et al., 1998), the role of the mGluRs is less clear. Inhibition of mGluR1 appears to be neuroprotective (Faden et al., 2001; Nicoletti et al., 1999). However, activation of Group I mGluRs may either protect or exacerbate injury depending upon the model and type of cell death (Allen et al., 2000).

Multiple lines of evidence suggest that Group I mGluRs can modulate NMDA receptor activity through several potential mechanisms: PKC-mediated enhancement of NMDA receptor activity, or changes in the polymerization state of the actin cytoskeleton via alterations in $[Ca^{2+}]_i$, or through a calcium/calmodulin pathway (Anwyl, 1999; Ehlers et al., 1996; Hisatsune et al., 1997; Holohean et al., 1999; Krupp et al., 1999; Rosenmund and Westbrook, 1993; Zhang et al., 1998). Moreover, Group I mGluR1 and mGluR5 subtypes may differentially utilize these mechanisms to affect changes in NMDA receptor current (Kawabata et al., 1996, 1998; Ugolini et al., 1997, 1999).

An in vitro neuronal stretch injury model has been used to study NMDA receptor modulation after trauma. Previous characterization of this model showed a significant correlation between amplitude of injury and the release of lactose dehydrogenase (LDH) or the uptake of propidium iodide, the latter being indicators of neuronal cell death (Ellis et al., 1995). Studies using this model found that a mild level of stretch causes an enhancement of NMDA receptor current and a reduced voltage-dependent Mg^{2+} block in neurons grown in the presence of glia (Zhang et al., 1996). In light of the above observations, we investigated whether there are correlations among the stretch-induced increases in NMDA receptor current, increasing amplitudes of stretch, and the release of LDH. Zhang et al. (1996) also showed that treatment with the PKC inhibitor, calphostin C, partially reversed the stretch-mediated reduction in the voltage-dependent Mg^{2+} block. Because Group I mGluR activation results in the upregulation of PKC through a phospholipase C pathway, we also examined the effect of modulating one Group I mGluR subtype (mGluR5) on stretch-induced enhancement of NMDA receptor current. Studies investigating effects of various compounds on either the voltage-dependent Mg^{2+} block of the NMDA receptor, or NMDA receptor current, have differed according to whether experiments were performed in either the presence or absence of extracellular magnesium. As there have been differing results with regard to the effects of mGluR modulation of NMDA receptor activity, we examined whether such differences may be due to the levels of $MgCl_2$ present. Moreover, because it has been suggested that certain modulatory affects of mGluR5 may result from actions in glial cells (Nicoletti et al., 1999), we also compared the affects of stretch on NMDA receptor current in cultures consisting of neurons grown in the absence or presence of a monolayer of glia.

2. Materials and methods

2.1. Drugs

The following drugs were obtained from Tocris Cookson (St. Louis, MO): *selective Group I mGluR agonist*—(*S*)-3,5-dihydroxyphenylglycine (DHPG); *selective mGluR5 agonist*—(*R*,*S*)-2-chloro-5-hydroxyphenylglycine (CHPG); *selective mGluR1 antagonist*—7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester (CPCCoEt); *selective mGluR5 antagonists*—(*R*,*S*)-1-aminoindan-1,5-darcarboxylic acid (AIDA), 2-methyl-6-(phenylethynyl) pyridine (MPEP); *L-type calcium channel blocker*—1, 4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-methyloxyethyl 1-methylethyl ester (nimodipine); *NMDA receptor agonist*—NMDA. The reversible sodium channel blocker, tetrodotoxin (TTX), was obtained from Sigma (St. Louis, MO). All drugs were prepared and stored according to the manufacturer's guidelines.

Selection of doses used for the mGluR5 compounds was based upon published EC_{50} and IC_{50} values (Schoepp et al., 1999), in addition to previous experience by this laboratory (Movsesyan et al., 2001; O'Leary et al., 2000) as well as others (Ugolini et al., 1999).

2.2. Neuronal–glial cultures

Glia were prepared from 1- to 3-day-old Sprague–Dawley rat cortices (Taconic Farms, Germantown, NY) and neurons were prepared from 17- to 18-day-old Sprague–Dawley rat embryonic cortices. For both glial and neuronal cultures, cortical hemispheres were isolated and minced in Krebs–Ringer bicarbonate buffer containing 0.3% bovine serum albumin (BSA; Life Technologies, Gaithersburg, MD). The cells were dissociated in 1800 U/ml trypsin (Sigma) at 37 °C for 20 min. Following dissociation, trypsinisation was halted by the addition of 200 U/ml DNase I and 3600 U/ml soybean trypsin inhibitor (Sigma). Individual cells were obtained by trituration and subsequent centrifugation through a 4% BSA layer. Glia were seeded in six-well silastic membrane stretch plates (Flex I Untreated Culture Plates; Flexcell International Hillsborough, NC) treated with poly-D-lysine and allowed to grow to confluency in minimal essential medium (MEM) with Earle's salts sans glutamine supplemented with 10% fetal bovine serum, 10% horse serum, 2.5% 1 M HEPES (pH 7.2), 1% 200 mM glutamine stock, 1% 2 M glucose stock, and 1% antibiotic–antimycotic. Neuronal cell suspension volume was adjusted with Hank's balanced salt solution without calcium or magnesium (Mediatech-CellGro, Herndon, VA) to $2–2.5 \times 10^6$ cells/ml and then diluted to 1:10 immediately before plating with neuronal seeding media [NSM; MEM with Earle's salts sans glutamine supplemented with 5% fetal bovine serum, 5% horse serum, 2.5% 1 M HEPES (pH 7.2), 1% 200 mM glutamine stock, 1% 2 M glucose stock,

and 1% antibiotic–antimycotic]. Neurons were plated onto the confluent layer of glial cells (DIV 10). Cultures were fed twice per week by replacement of one-half of media with MEM with Earle's salts (Mediatech-CellGro, Herndon, VA) supplemented with 10% equine serum, 2.5% 1 M HEPES (pH 7.2), 1% 200 mM glutamine (Biofluids, Rockville, MD), 1% 2 M glucose (Biofluids, Rockville, MD), and 1% antibiotic–antimycotic solution (Biofluids Rockville, MD; feeding solution A). Cytosine-β-D-arabinofuranoside (10 μM; Sigma) was added during the first feeding to stop further glial proliferation (feeding solution B). Subsequent feedings, alternating between feeding Media A and B, were done twice per week until cells were used (14–25 DIV). Cell cultures were maintained at 37 °C in humid atmosphere with 5% CO_2.

2.3. Neuronal cultures

Cortical neurons were derived similar to the methods outlined above. Following centrifugation through the 4% BSA layer, however, the cell pellet was resuspended in NSM consisting of Neurobasal Medium (Invitrogen-Gibco, Carlsbad, CA), supplemented with 1.1% 100× antibiotic–antimycotic solution (Biofluids, Rockville, MD), 25 μM Na-glutamate, 0.5 mM L-glutamine, and 2% B27 Supplement (Invitrogen-Gibco, Carlsbad, CA). Cells (2.5×10^5 cells/ml) were seeded onto six-well silastic membrane stretch plates (Flex I Untreated Culture Plates; Flexcell International) pretreated with poly-D-lysine. On Day 4, the seeding media was replaced with feeding media (NSM without Na-glutamate and B27 supplement) in a 1:2 proportion. Subsequent feedings using the same feeding media were done twice per week until cells were used (14–25 DIV). Cell cultures were maintained at 37 °C in humid atmosphere with 5% CO_2.

2.4. Stretch model and electrophysiology

Cells cultured upon a deformable membrane can be stretched with compressed gas at known durations and pressures equating to varying levels of injury (Ellis et al., 1995). On the day of the experiment, growth media was replaced by a recording solution consisting of: 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM MgCl, 5 mM HEPES, 5 mM glucose, and 2 mM glycine. Osmolarity was adjusted to 325 mOsm with sucrose, and pH adjusted to 7.4 with NaOH prior to media substitution. After switching the media, cells were returned to the incubator for at least 20 min prior to injury in the absence or presence of various Group I mGluR group- and subtype-specific compounds. Stretch (5.7 or 7.5 mm deformations of the membrane; 50 ms duration) was applied to the cells using a Cell Injury Controller (Biomedical Engineering Facility, Medical College of Virginia). Following the stretch, cells were returned to the incubator for an additional hour prior to recording. At the time of recording, a portion of the silastic membrane was transferred to a recording chamber undergoing continuous perfusion of recording solution on the stage of an inverted microscope (Zeiss Axiovert 135) . Electrophysiological recordings were performed at room temperature (20–22 °C). Electrodes were pulled from a Wiretrol II capillary glass (Drummond Scientific, Broomall, PA) in three stages on a horizontal pipette puller (Mecanex, Nyon, Switzerland). Typical pipette resistance was 4–11 MΩ. The recording pipette contained (mM) 145 K-gluconate, 5 $MgCl_2$, 11 ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 Na-adenosine-5′-triphosphate (ATP), 0.2 guanosine-5′-triphosphate (GTP), and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.2 with KOH. Whole cell recordings were performed with a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA) after capacitance and series resistance compensation. Cultured cortical neurons were voltage-clamped at a −60 mV holding potential. Access and input resistances were monitored intermittently during recordings by giving a 10-mV hyperpolarizing pulse. Application of drugs to the cells was via Y tubing controlled by a BPS-4 valve control system (Scientific Instruments, New York, NY). The recording solution (see above) used for wash and for drug application was supplemented with 10 μM nimodipine and 0.6 μM TTX. Electrophysiological recordings were both digitized and analyzed using pClamp 8 software (Axon Instruments).

2.5. Assays

Stretch injury-induced release of LDH was measured 24 h postinjury. Growth media was replaced with extracellular recording solution (ERS; see above) prior to mild and severe stretch injury. One six-well stretch plate was used for each treatment group (control, 5.7 mm stretch, 7.5 mm stretch). Twenty-four hours poststretch injury (see above), aliquots of ERS were removed from each well ($n = 6$) and frozen (−20 °C) until assayed. LDH was measured using a CytoTox-96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Relative absorbance was measured at 490 nm using a Multiskan *Ascent* microplate reader (Labsystems Oy, Helsinki, Finland). Following the subtraction of background LDH levels measured in uninjured cells, 5.7- and 7.5-mm injury-induced LDH levels were compared.

2.6. Data analysis

Data were analyzed using Clampfit 8.0 (Axon Instruments), Excel (Microsoft Corp., Redmond, WA), and Statview 5.0.1 (SAS Institute, Cary, NC). Studies were performed in cortical neurons cultured in the presence (NG) or absence (PN) of a monolayer of glial cells. All currents were measured at the peak of the response. Average cell capacitance (pF) was estimated from the transient

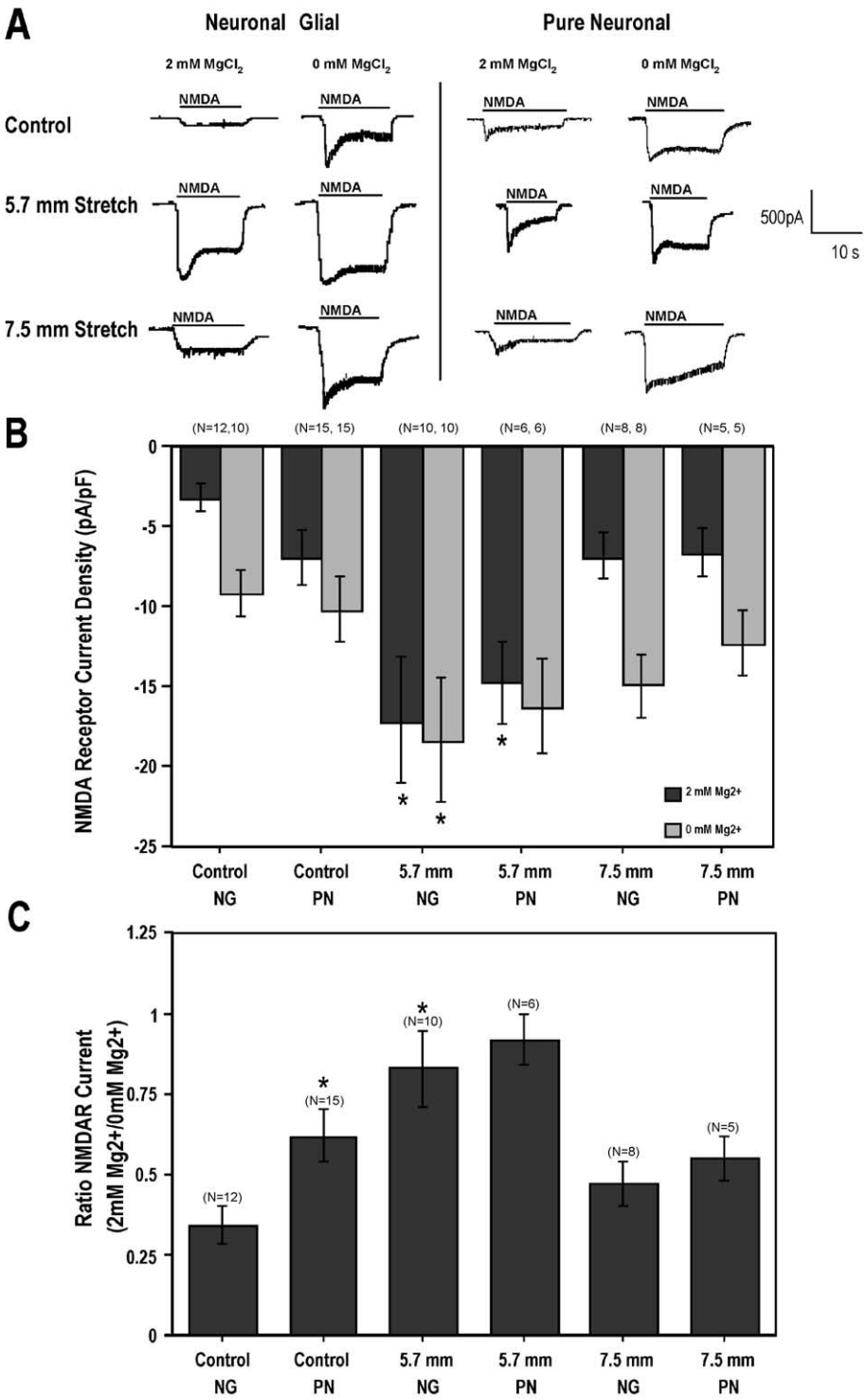

Fig. 1. Effects of stretch injury on NMDA receptor activity. (A) Representative traces of NMDA-evoked whole cell currents in the absence of stretch injury, and after 5.7 and 7.5 mm stretch of the silastic membrane. Whole cell currents were recorded from 14 to 25 DIV cultured cortical neurons voltage clamped at − 60 mV. (B) The 5.7-mm, but not 7.5-mm, stretch significantly increased the current through the NMDA receptor in both the presence ($P < .05$; ANOVA) and absence ($P < .05$; ANOVA) of 2 mM extracellular $MgCl_2$ and in both rat cortical neurons cultured on top of a monolayer of glial cells (neuronal glial; NG) and in the absence of the glial monolayer (pure neuronal; PN). (C) In NG neurons, the 5.7-mm stretch significantly increased the ratio of I_{NMDA} measured in the presence to that measured in the absence of 2 mM extracellular $MgCl_2$ ($P < .05$; ANOVA). The ratio in PN neurons did not reach significance ($P > .05$; ANOVA). Ratios in control PN neurons were significantly different from control NG neurons ($P < .05$; ANOVA). Ratios in both NG and PN neurons were similar to those measured in control after the 7.5-mm stretch ($P > .05$; ANOVA). * Significant differences from NG controls.

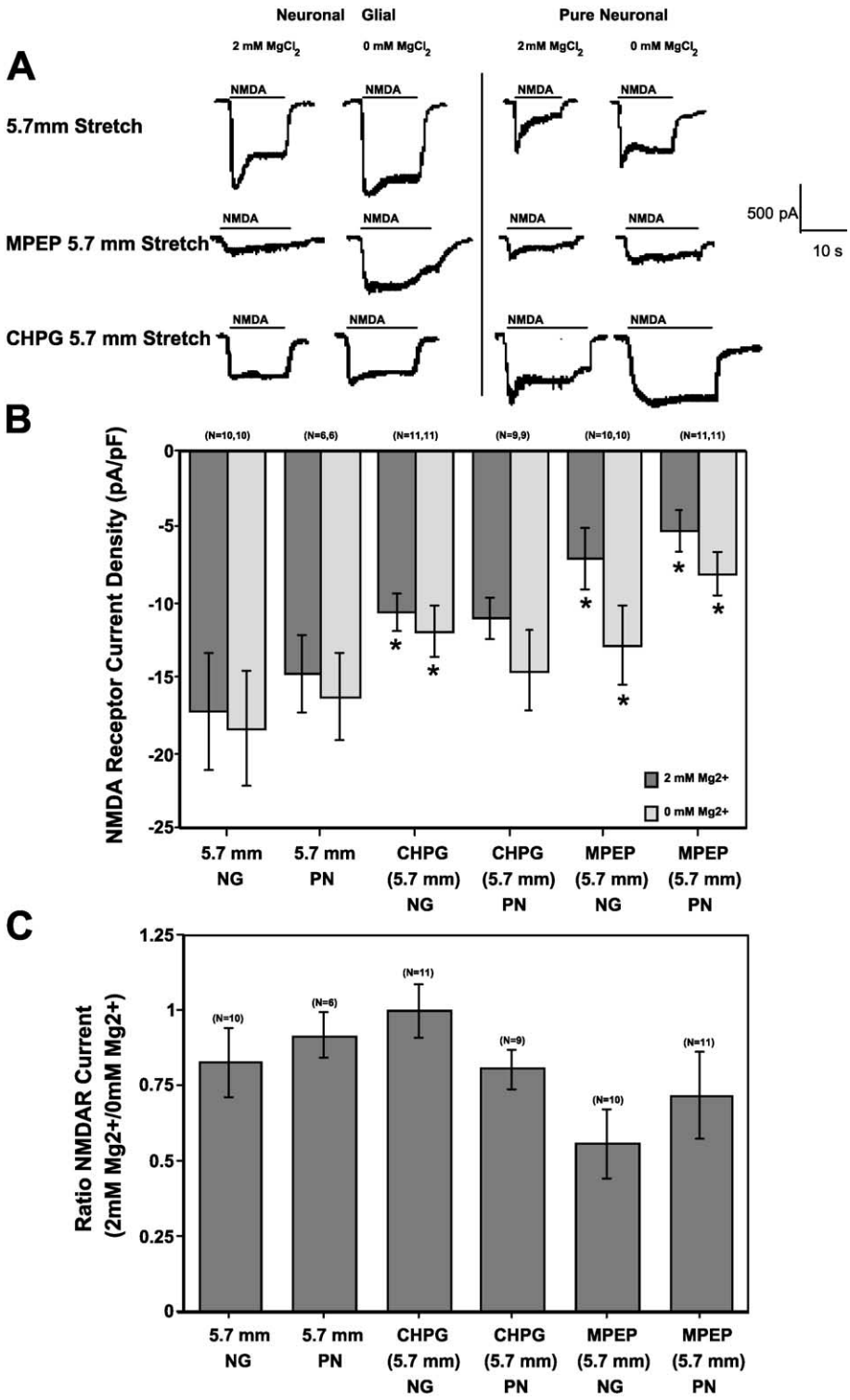

Fig. 2. Effects of mGluR5 modulation prior to the 5.7-mm stretch injury. (A) Representative traces of NMDA-evoked whole cell currents measured after the 5.7-mm stretch with and without pretreatment using either the mGluR5 antagonist, MPEP (2 μM), or the agonist, CHPG (1 mM). Whole cell currents were recorded from 14 to 25 DIV cultured cortical neurons voltage clamped at − 60 mV. (B) Pretreatment of NG , but not PN, cortical neurons with the mGluR5 agonist, CHPG (1 mM), caused a significant reduction in the enhanced NMDA inward current seen after the 5.7-mm stretch ($P < .05$; ANOVA). Pretreatment of both NG and PN cortical neurons with the mGluR5 antagonist, MPEP (2 μM), caused a significant reduction in the enhanced NMDA inward current seen after the 5.7-mm stretch ($P < .05$; ANOVA). (C) Neither pretreatment with CHPG nor MPEP had any significant effect on the ratio of I_{NMDA} measured after the 5.7-mm stretch ($P > .05$; ANOVA), although significant differences were found between CHPG- and MPEP-treated NG neurons ($P < .05$; ANOVA). * Significant differences from the 5.7-mm stretched neurons.

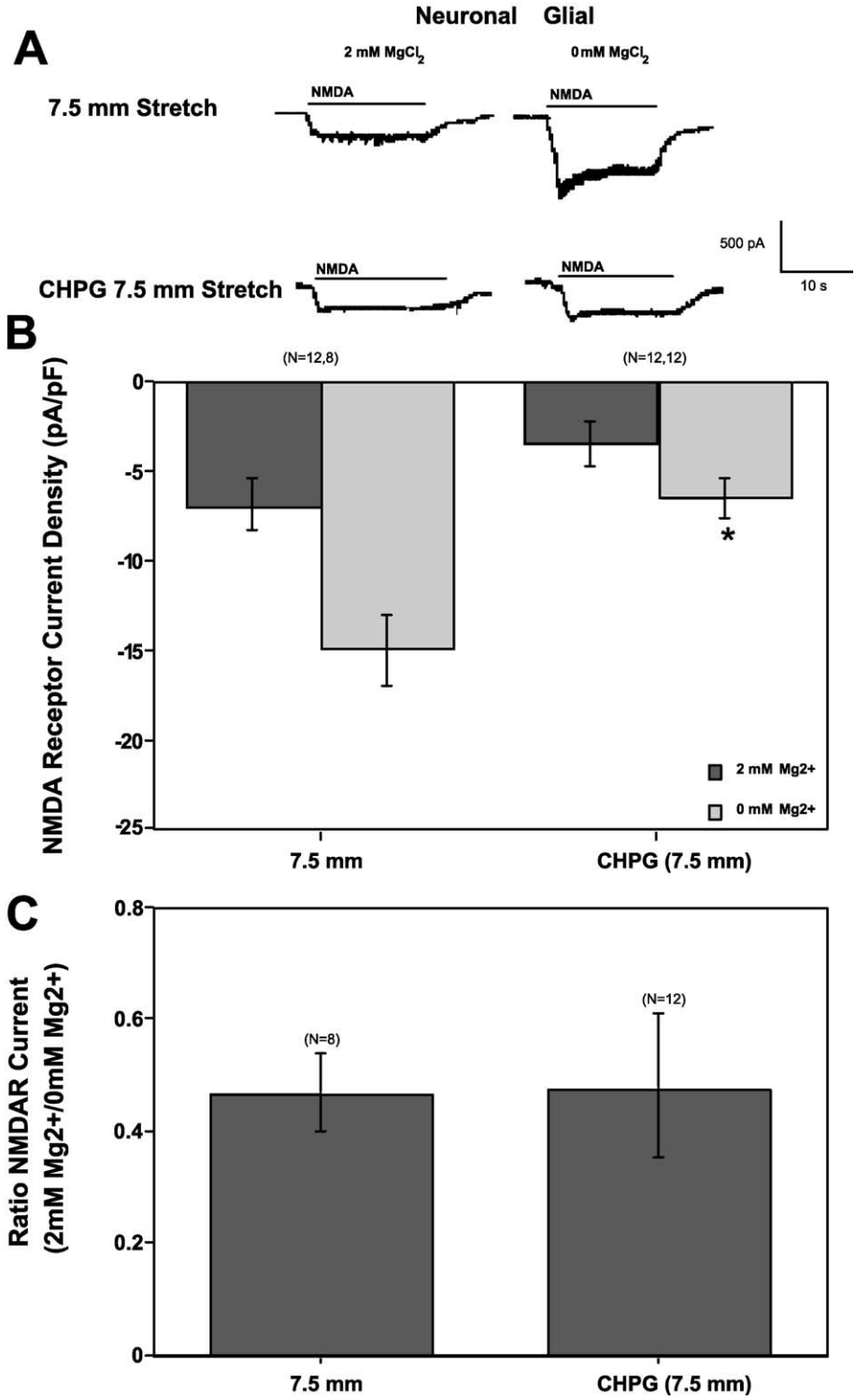

Fig. 3. Effects of the mGluR5 agonist, CHPG, on NMDA receptor current and current ratio in NG neurons following the 7.5-mm stretch. (A) Representative traces of NMDA-evoked whole cell currents measured after the 7.5-mm stretch of NG neurons with and without CHPG (1 mM) pretreatment. Whole cell currents were recorded from 14 to 25 DIV cultured cortical neurons voltage clamped at -60 mV. (B) Pretreatment of NG cortical neurons with the mGluR5 agonist, CHPG (1 mM), caused a significant reduction in NMDA receptor inward current measured in the absence of 2 mM extracellular $MgCl_2$ ($P<.05$; ANOVA), but not in the presence of 2 mM extracellular $MgCl_2$ ($P>.05$; ANOVA). (C) Pretreatment of NG cortical neurons with the mGluR5 agonist, CHPG (1 mM), prior to the 7.5-mm stretch did not significantly change the ratio of I_{NMDA} from levels measured after the 7.5-mm stretch ($P>.05$; ANOVA). * Significant difference from current density measured in the 7.5-mm stretched neurons in the absence of 2 mM extracellular Mg^{2+}.

relaxation currents produced by the 10-mV hyperpolarizing voltage pulses given at the beginning of recording in each cell (Corsi et al., 1998). NMDA receptor currents (pA) were normalized to cell capacitance (pF) to provide a measure of current density (pA/pF). Current densities (pA/pF) and the 2 mM Mg^{2+}/0 mM Mg^{2+} ratios were analyzed using a one-way ANOVA followed by Fischer's PLSD. Significance was accepted at an $\alpha < .05$.

3. Results

3.1. Stretch injury, NMDA receptor activity, and cell death

NMDA receptor current density (I_{NMDA}), determined by the responses to application of 200 μM NMDA in both the presence and absence of 2 mM extracellular $MgCl_2$, was similar (Fig. 1B; $P>.05$; ANOVA) in both NG cortical neurons (-3.25 pA/pF $\pm$ 0.87 S.E.M., -9.17 ± 1.48 S.E.M., respectively; $n=12$) and PN cortical neurons (-6.95 pA/pF $\pm$ 1.85 S.E.M., -10.21 ± 1.98 S.E.M., respectively; $n=15$). The 5.7-mm stretch significantly enhanced I_{NMDA} (Fig. 1B; $P<.05$; ANOVA) in the presence of 2 mM extracellular $MgCl_2$ in both NG cortical neurons (-17.10 pA/pF $\pm$ 3.97 S.E.M.) and PN cortical neurons (-15.08 pA/pF $\pm$ 3.03 S.E.M.). In the absence of 2 mM extracellular $MgCl_2$, however, significant increases in I_{NMDA} following the 5.7-mm stretch were observed in NG cortical neurons (-18.34 ± 3.86 S.E.M.; $n=10$; $P<.05$; ANOVA), but not PN cortical neurons (-16.27 pA/pF $\pm$ 2.91 S.E.M.; $n=6$; $P>.05$; ANOVA; Fig. 1B). A 7.5-mm stretch injury had no effect on I_{NMDA} (Fig. 1B; $P>.05$; ANOVA) in either NG cortical neurons (-6.88 pA/pF $\pm$ 1.46 S.E.M., -14.93 ± 1.94 S.E.M., respectively; $n=8$) or PN cortical neurons (-6.63 pA/pF $\pm$ 1.46 S.E.M., -12.33 ± 2.04 S.E.M., respectively; $n=5$), whether or not $MgCl_2$ (2 mM) was present. Responses due to activation of the NMDA receptor after stretch injury did not have consistently altered profiles; however, large response variability was found between cells in all paradigms.

The absence of a monolayer of glia caused a significant difference between the ratios of NMDA receptor inward currents measured in 2 mM extracellular $MgCl_2$ to inward currents measured in 0 mM extracellular $MgCl_2$ (2 mM Mg^{2+}/0 mM Mg^{2+} ratios) observed in control NG and PN cortical neurons (0.34 ± 0.06 S.E.M., $n=12$; 0.62 ± 0.08 S.E.M., $n=15$; respectively; $P<.05$; ANOVA; Fig. 1C). A mild 5.7-mm stretch

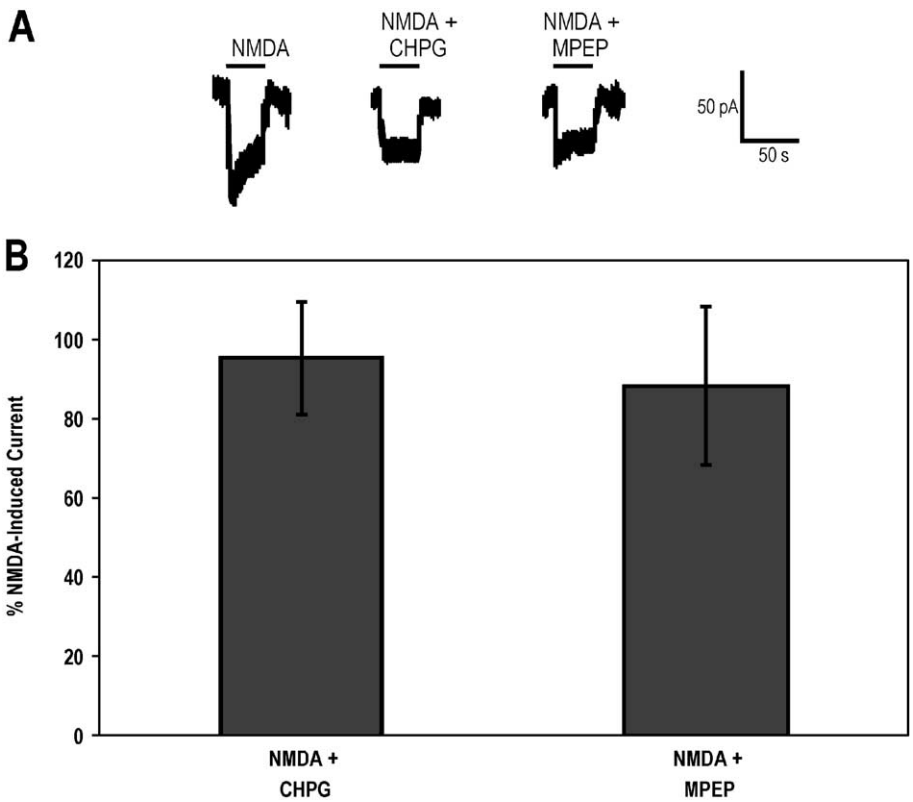

Fig. 4. Effects of CHPG and MPEP on NMDA-induced inward current in NG cortical neurons. (A) Representative traces of NMDA (200 μM)-induced inward current with or without coapplication of the mGluR5 agonist, CHPG (1 mM), or the mGluR5 antagonist, MPEP (2 μM). (B) Normalized to NMDA-induced currents, inward currents induced by NMDA (200 μM) and either CHPG (1 mM; $n=7$) or MPEP (2 μM; $n=6$) show no significant effects of these mGluR5-specific compounds on NMDA-evoked inward current ($P>.05$; ANOVA).

significantly increased the 2 mM Mg^{2+}/0 mM Mg^{2+} ratios from control values in NG cortical neurons (0.83 ± 0.12 S.E.M.; $n = 10$; $P < .05$; ANOVA), but not in PN cortical neurons (0.92 ± 0.08 S.E.M.; $n = 6$; $P > .05$; ANOVA; Fig. 1C). A more severe 7.5-mm stretch did not significantly alter the 2 mM Mg^{2+}/0 mM Mg^{2+} ratios from control values in either NG (0.47 ± 0.07 S.E.M.; $n = 8$) or PN (0.55 ± 0.07 S.E.M.; $n = 5$) cortical neurons (Fig. 1C; $P > .05$; ANOVA). In PN neurons, stretch injury caused an injury dose-dependent release of LDH. Injury-induced LDH measured 24 h postinjury was 360 ± 124% (S.E.M.) greater in 7.5-mm stretched neurons as compared to levels measured after the 5.7-mm stretch (taken as 100%).

3.2. Modulation by mGluR5 compounds

Pretreatment with the mGluR5 antagonist, MPEP, limited the 5.7-mm stretch-induced increases in I_{NMDA} measured in the presence or absence of 2 mM extracellular $MgCl_2$ in both NG (−7.06 ± 1.99 S.E.M., −12.76 ± 2.61 S.E.M., respectively; $n = 10$) and PN (−5.51 ± 1.49 S.E.M., −8.00 ± 1.47 S.E.M., respectively; $n = 11$) cortical neurons (Fig. 2B; $P < .05$; ANOVA). I_{NMDA} measurements were similar to control levels ($P > .05$; ANOVA). In contrast, pretreatment with the mGluR5 agonist, CHPG (1 mM), limited the 5.7-mm stretch-induced increases in I_{NMDA} measured in the presence or absence of 2 mM extracellular $MgCl_2$ in NG (−10.79 ± 1.41 S.E.M., −11.83 ± 1.75 S.E.M., respectively; $n = 11$; $P < .05$; ANOVA), but not PN (−10.96 ± 1.49 S.E.M., −14.46 ± 2.69 S.E.M., respectively; $n = 9$; $P > .05$; ANOVA) cortical neurons (Fig. 2B). CHPG reduced the 5.7-mm stretch-induced increases in I_{NMDA} measured in the absence, but not presence, of 2 mM extracellular $MgCl_2$ back to control levels ($P > .05$; ANOVA). Neither MPEP, nor CHPG, had any significant effect on the 5.7-mm stretch-induced increases in the 2 mM Mg^{2+}/0 mM Mg^{2+} ratios measured in both NG (0.56 ± 0.12 S.E.M., $n = 10$; 1.00 ± 0.09 S.E.M., $n = 11$; respectively) and PN (0.72 ± 0.15 S.E.M., $n = 11$; 0.81 ± 0.07 S.E.M., $n = 9$; respectively) cortical neurons (Fig. 2C; $P > .05$; ANOVA), although, in both culture conditions, MPEP appears to reduce the ratio back towards control levels ($P > .05$; ANOVA). Pretreatment with CHPG prior to the 7.5-mm stretch had no significant effect on I_{NMDA} measured in the presence of 2 mM extracellular $MgCl_2$ in NG cortical neurons (−3.43 ± 1.18 S.E.M., $P > .05$; ANOVA; Fig. 3B). In contrast, CHPG did reduce I_{NMDA} measured in the absence of 2 mM extracellular $MgCl_2$ in NG cortical neurons (6.47 ± 1.04 S.E.M.; $n = 12$; $P < .05$; ANOVA). However, the data were not significantly different from controls ($P > .05$; ANOVA). Pretreatment with CHPG prior to the 7.5-mm stretch had no significant effect on the 2 mM Mg^{2+}/0 mM Mg^{2+} ratio in NG cortical neurons (0.48 ± 0.13 S.E.M.; $n = 12$; $P > .05$; ANOVA; Fig. 3C).

3.3. Direct effects of Group I mGluR compounds on NMDA receptor activity

Fig. 4 shows that when coapplied under saturating conditions (i.e., 200 μM NMDA and 2 μM glycine) in the presence of 2 mM extracellular $MgCl_2$, neither 2 μM MPEP ($n = 6$) nor 1 mM CHPG ($n = 7$) had any significant effects on NMDA-evoked I_{NMDA} in our NG cortical neurons ($P > .05$; ANOVA). The amplitude of the responses to NMDA alone (Fig. 4) was comparable with the NG control traces in Fig. 1A measured in the presence of 2 mM extracellular $MgCl_2$.

4. Discussion

Multiple lines of evidence show that NMDA receptors are intimately involved in the process of cell death following traumatic injury (for a review, see Temple et al., 2001). In addition to activation of the NMDA receptor by trauma-induced release of glutamate, injury-induced enhancement of NMDA receptor activity can occur by modulatory changes such as a reduced voltage-dependent Mg^{2+} block of the NMDA receptor, a change in the expression of NMDA receptors, an increased peak open probability, or a change in receptor subtype expression with different open probabilities (Chen et al., 1999). The latter three possibilities reflect a change in NMDA current density. Under normal resting conditions, the maintenance of the NMDA receptor voltage-dependent Mg^{2+} block prevents the inward movement of extracellular calcium through the NMDA receptor pore. When this Mg^{2+} block is relieved, either through a positive change in membrane potential or through a modification of the receptor by endogenous effectors, the inward movement of calcium can have profound effects, such as enhanced release of neurotransmitter or the induction of cell death.

An in vitro stretch trauma model developed by Ellis et al. (1995), thought to parallel the stretch forces generated during diffuse axonal injury (Tomei et al., 1990), is well suited for studying trauma-induced changes in NMDA receptor activity. Previous use of this model using various cell types has demonstrated significant alterations in membrane, receptor, and mitochondrial properties, which can lead to the activation of both necrotic and apoptotic pathways (Ahmed et al., 2000; Ellis et al., 1995; Goforth et al., 1999; Hoffman et al., 2000a,b; Lamb et al., 1997; Pike et al., 2000; Rzigalinski et al., 1997, 1998; Tavalin et al., 1997; Weber et al., 1999, 2001; Zhang et al., 1996). One such study showed that mild injury to rat cortical neurons, grown in coculture with glia, caused an enhancement of NMDA-evoked inward current via a reduction of the NMDA receptor voltage-dependent Mg^{2+} block (Zhang et al., 1996). This conclusion was supported by whole cell patch recordings of NMDA receptor currents measured while sequentially changing the cell membrane potential: cur-

rent–voltage (I–V) relationships showed that at +40 mV, there was no difference in NMDA inward current between control and stretched neurons. Taken together, their results indicate that mild stretch injury has no affect on maximal NMDA receptor current and, therefore, stretch-induced changes in NMDA receptor current are due to the reduction of the voltage-dependent Mg^{2+} block and not alterations in NMDA current density.

The above findings, combined with studies showing that activation of NMDA receptors is intimately involved with cell death (Mukhin et al., 1997a,b), lead us to the hypothesis that increases in levels of stretch injury would cause a corresponding increase in NMDA receptor activity. To test this hypothesis, we compared the effects of a mild (5.7 mm) and severe (7.5 mm) stretch injury on NMDA receptor current density, as well as on the ratio of NMDA receptor inward currents measured in 2 versus 0 mM extracellular $MgCl_2$. In the original work characterizing the stretch model, the terms 'mild,' 'moderate,' and 'severe' were used to define 5.5, 6.5, and 7.5 mm levels of stretch, respectively (Ellis et al., 1995). The 2 mM Mg^{2+}/0 mM Mg^{2+} ratio can provide an indication of the extent of the NMDA receptor voltage-dependent Mg^{2+} block so long as the alterations measured at a −60 mV holding potential are physiological and not due to possible errors in voltage clamping at one potential. Zhang et al. (1996) used standard current–voltage relationships to demonstrate that at a −60 mV holding potential stretch-induced enhancement of NMDA receptor is physiological. In support of their findings, we found that a mild level of stretch injury to rat cortical neurons, grown in the presence or absence of a monolayer of glia, and in the presence of extracellular $MgCl_2$ induced significant increases NMDA receptor activity and reduced the voltage-dependent Mg^{2+} block. In contrast, however, we observed significant increases in NMDA receptor current measured in the absence of extracellular $MgCl_2$, suggesting that in addition to the reduced voltage-dependent Mg^{2+} block, mild stretch injury also increases maximal NMDA receptor current through alterations in NMDA current density. These findings are supported by previous studies showing injury-induced changes in NMDA receptor subunit composition (Kreutz et al., 1998; Sun and Faden, 1995). Potential explanations for the differences between our findings and those of Zhang et al. (1996) include differences in culture conditions (Condorelli et al., 1993; Daniels and Brown, 2001) and the age of the cells (Zhong et al., 1994). Both of these conditions can influence the expression pattern of the NMDA receptor subtypes. Additionally, the age of the cells can influence the coupling of mGluR and iGluR to different phosphorylation systems (Angenstein et al., 1999).

In contrast to the 5.7-mm stretch injury, increasing the level of stretch to a more severe level (7.5 mm) had no effect on either the inward current or voltage-dependent Mg^{2+} block of the NMDA receptor, despite more elevated levels of LDH measured 24 h postsevere injury. These results suggest that the increased levels of LDH at higher levels of stretch do not reflect NMDA receptor-dependent cell death. It is possible that more severe stretch injury may cause calcium-dependent desensitization of the NMDA receptor (for a review, see McBain and Mayer, 1994), or inhibition of NMDA receptor activity by injury-induced release of redox-related congeners of nitric oxide or anandamide (Hampson et al., 1998; Kim et al., 1999; Lipton and Stamler, 1994). A possibility remains that at higher levels of stretch, cell death may be primarily due to other factors, such as activation of calpain and/or caspase-3 (Pike et al., 2000). Because the goal of this study was to look at stretch-enhanced NMDA receptor activity, we focused on the 5.7-mm model of injury for the pharmacological studies.

Group I mGluR activation has been shown to modulate NMDA receptor activity in multiple in vitro systems (Allen et al., 2000; Awad et al., 2000; Bortolotto and Collingridge, 1995; Colwell et al., 1996; Contractor et al., 1998; Holohean et al., 1999; Kinney and Slater, 1993; Skeberdis et al., 2001; Ugolini et al., 1997, 1999; Yu et al., 1997), suggesting a likely role for Group I mGluR-mediated enhancement of NMDA receptors in excitotoxicity following neuronal injury. Indeed, in mouse cortical neurons, Bruno et al. (1995, 1999) showed that Group I mGluR agonists amplify NMDA-induced neuronal degeneration through PKC activation and that LY367385, a potent and selective antagonist of mGluR1a, was neuroprotective in cultures exposed to an NMDA pulse. In rat cortical/glial cocultures, Mukhin et al. (1996, 1997b) showed that Group I mGluR activation exacerbates in vitro punch injury-induced delayed neuronal cell death and that MK801 partly reduced injury exacerbation caused by the Group I mGluR agonist, DHPG.

To test the prediction that mGluR5 modulates both mild (5.7 mm) stretch-induced increases in NMDA receptor activity and reductions in the voltage-dependent Mg^{2+} block of the NMDA receptor, we pretreated our cells with either the mGluR5 agonist, CHPG (1 mM), or antagonist, MPEP (2 μM), prior to injury. MPEP limited mild stretch-induced changes in NMDA current density and the voltage-dependent Mg^{2+} block in cortical neurons grown in the presence or absence of a glial monolayer. These findings are consistent with studies showing mGluR5-mediated enhancement of NMDA receptor activity (Awad et al., 2000; Ugolini et al., 1999), and a reciprocal positive feedback interaction between these two glutamate receptor subtypes (Alagarsamy et al., 1999). Previous data from this laboratory (O'Leary et al., 2000), as well as others (Contractor et al., 1998), suggest that the reduced NMDA receptor inward current may be due to direct interactions of MPEP on the NMDA receptor. However, we do not believe this to be the case here, as all of our experiments utilized saturating concentrations of NMDA and glycine. In addition, neither MPEP (2 μM) nor CHPG (1 mM) had any effects on NMDA-evoked inward currents in cells not subjected to stretch.

Surprisingly, the mGluR5 agonist, CHPG, also prevented mild stretch-induced increases in NMDA current density in cortical neurons cultured in the presence of a monolayer of glia. These findings are in contrast to the studies noted above, in which mGluR5 activation enhanced NMDA receptor currents in subthalamic nucleus (Awad et al., 2000) and spinal cord (Ugolini et al., 1999) neurons. It may be that these differences are dependent upon the source of neurons for culture (Bruno et al., 1996, 1998), or due to pretreating with the mGluR5 agonist prior to injury. Studies show that activation of mGluR5 can lead to rapid desensitization of the receptor (Gereau and Heinemann, 1998), or a switch from 'neurotoxic' to 'neuroprotective' activity (Bruno et al., 2001). However, we believe that the ability of CHPG to exert its effects is predominately due to glial mGluR5 receptors, as pretreatment with CHPG had no effect on mild stretch-induced increases in NMDA current density in our cultured neurons grown in the absence of a glial monolayer. Although it is possible that the presence of the monolayer of glia alone significantly alters the NMDA receptor expression in such a way as to make the two culture conditions noncomparable (Daniels and Brown, 2001), a statistical comparison between mean NMDA receptor currents of the two populations argues against such a possibility. Combined, our data suggest a significant role for neuronal mGluR5 receptors in regulating mild stretch-induced enhancement of NMDA current density, and a significant role for glial mGluR5 receptors in regulating neuronal NMDA current density.

Previous reports from this laboratory, as well as others, show that Group I mGluR compounds can directly affect the NMDA receptor (Contractor et al., 1998; Movsesyan et al., 2001; O'Leary et al., 2000). It is postulated that this may be due to the use of low concentrations of NMDA and glycine (Contractor et al., 1998), or to the concentration of extracellular Mg^{2+}. Indeed, a previous study showed that the modulatory effect of the Group I/II mGluR agonist, *trans*-ACPD, on NMDA receptor activity in frog spinal cord is dependent upon extracellular Mg^{2+} concentration (Holohean et al., 1999). We show that the presence or absence of extracellular magnesium influences whether the observed mGluR-induced enhancement of NMDA receptor activity occurs through alterations in the voltage-dependent Mg^{2+} block of the NMDA receptor, or through changes in NMDA current density.

As discussed, neither MPEP nor CHPG significantly altered NMDA-evoked inward currents measured in the presence of $MgCl_2$ in cells not subjected to stretch injury. These results suggest that the effects of the mGluR compounds were not due to direct interactions with the NMDA receptor. Combined, our results indicate that inhibition of neuronal mGluR5 prior to stretch injury reduces injury-induced increases in maximal NMDA-evoked inward currents by significantly altering NMDA current density and by limiting the stretch-induced reductions of the voltage-dependent Mg^{2+} block of the NMDA receptor. Furthermore, our data suggest that activation of glial mGluR5 prior to stretch injury reduces stretch-induced increases in maximal NMDA-evoked inward currents by significantly altering neuronal NMDA current density through an as yet unidentified neuronal/glial interaction. Although glial mGluR5 activation did not significantly alter the stretch-reduced voltage-dependent Mg^{2+} block of the NMDA receptor, there appeared to be a trend for CHPG to exacerbate stretch-induced reductions in the 2 mM Mg^{2+}/0 mM ratios. The ability of glial mGluR5 to regulate the voltage-dependent Mg^{2+} block of neural NMDA receptors is also supported by our data, as we found a significant difference between the 2 mM Mg^{2+}/0 mM ratios measured after CHPG and MPEP in the cocultures. These data suggest that studies looking at the protective/excitotoxic effects of mGluR5, particularly in relation to NMDA receptor-induced cell death, need to differentiate between the effects due to activation of glial and/or neuronal mGluR5.

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