Activation of mGluR5 and Inhibition of NADPH Oxidase Improves Functional Recovery after Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) induces microglial activation, which can contribute to secondary tissue loss. Activation of mGluR5 reduces microglial activation and inhibits microglial-mediated neurodegeneration *in vitro*, and is neuroprotective in experimental models of CNS injury. *In vitro* studies also suggest that the beneficial effects of mGluR5 activation involve nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibition in activated microglia. We hypothesized that activation of mGluR5 by the selective agonist CHPG after TBI in mice is neuroprotective and that its therapeutic actions are mediated by NADPH oxidase inhibition. Vehicle, CHPG, or CHPG plus the mGluR5 antagonist (MPEP), were administered centrally, 30 minutes post-TBI, and functional recovery and lesion volume was assessed. CHPG significantly attenuated post-traumatic sensorimotor and cognitive deficits, and reduced lesion volumes; these effects were blocked by MPEP, thereby indicating neuroprotection involved selective activation of mGluR5. CHPG treatment also reduced NF κ B activity and nitrite production in lipopolysaccharide-stimulated microglia and the protective effects of CHPG treatment were abrogated in NADPH oxidase deficient microglial cultures (gp91^{phox}^{-/-}). To address whether the neuroprotective effects of CHPG are mediated via the inhibition of NADPH oxidase, we administered the NADPH oxidase inhibitor apocynin with or without CHPG treatment after TBI. Both apocynin or CHPG treatment alone improved sensorimotor deficits and reduced lesion volumes when compared with vehicle-treated mice; however, the combined CHPG + apocynin treatment was not superior to CHPG alone. These data suggest that the neuroprotective effects of activating mGluR5 receptors after TBI are mediated, in part, via the inhibition of NADPH oxidase.

Key words: functional recovery; microglia; metabotropic glutamate receptor 5; NADPH oxidase; neuroprotection; traumatic brain injury

Introduction

CELL DEATH AND TISSUE LOSS after traumatic brain injury (TBI) reflect complex biochemical cascades resulting from the direct physical disruption of tissue (primary injury), as well delayed and potentially reversible molecular pathophysiological changes (secondary injury).¹ Secondary injury mechanisms include glutamate excitotoxicity, blood-brain barrier disruption, secondary hemorrhage and ischemia, mitochondrial dysfunction, apoptotic and necrotic cell death, and inflammation, among others.^{2,3} Secondary injury begins within sec to min after the primary insult and may continue for days, weeks, or months, contributing to progressive tissue loss. These processes are associated with infiltration and activation of blood-borne immune cells such as macrophages and lymphocytes, as well as activation of resident microglia that may promote chronic neurodegeneration.⁴

Metabotropic glutamate receptors (mGluR) are G-protein-coupled receptors that have been classified into three groups (I–III) based on sequence homology, signal transduction mechanism, and pharmacological profile.^{5–7} Within group I receptors, mGluR1 activation exacerbates neuronal death, particularly necrotic cell death.⁸ In contrast, the mGluR5 agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), is neuroprotective and has anti-apoptotic properties in neuronal cell culture models (amyloid- β (A β) and MPP⁺ induced neuronal toxicity),^{8–11} and also has strong anti-inflammatory effects in microglial cell culture models.^{12,13}

Selective activation of mGluR5 by CHPG has powerful neuroprotective properties in *in vivo* CNS injury models. For example, in a rat model of focal cerebral ischemia, CHPG administration reduced the infarct volume and improved neurological function at 24 hours after MCAO/reperfusion.¹⁴ CHPG treatment also improved motor function recovery, reduced lesion volume, and

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increased white matter sparing at 28 days after spinal cord injury (SCI) in rats.¹⁵ Notably, the protective effects of CHPG were mediated in part by a reduction in post-traumatic microglial activation in the injured spinal cord because treated animals had reduced markers of microglial activation (e.g., CD68 and galectin-3), reduced expression of pro-inflammatory mediators (e.g., tumor necrosis factor- α (TNF α) and inducible nitric oxide synthase (iNOS)), and reduced expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

NADPH oxidase is a multi-subunit enzyme complex responsible for the production of both extracellular and intracellular reactive oxygen species (ROS) by phagocytic cells including microglia. NADPH oxidase is composed of cytoplasmic subunits (p47^{phox}, p67^{phox}, p40^{phox}, and Rac2), which, on phosphorylation by specific kinases, can form a complex and translocate to dock with the membrane subunits (gp91^{phox} and p22^{phox}).¹⁶ NADPH oxidase has been implicated as a common and essential mechanism of microglia-mediated neurotoxicity.¹⁷ Activation of the NADPH oxidase enzyme complex in microglia is neurotoxic, both through the production of extracellular ROS that damage neighboring neurons as well as the initiation of redox signaling in microglia that amplifies the pro-inflammatory response, thereby maintaining microglia in a chronically activated and neurotoxic state.^{18,19}

We previously reported that CHPG treatment, acting through mGluR5, reduces microglial activation and the associated release of pro-inflammatory mediators in microglial cell culture models after stimulation with the classical activators such as lipopolysaccharide (LPS) or interferon- γ (IFN γ).^{12,13} CHPG treatment also reduced NADPH oxidase activity levels and abolished the neurotoxic potential of activated microglia in microglia/neuron co-culture models. The protective effects of CHPG were blocked by knockout of the mGluR5 or by addition of the selective mGluR5 antagonist MTEP, and reduced by co-incubation with siRNAs directed against either of the two membrane subunits of NADPH oxidase (p22^{phox} or gp91^{phox}).¹³ In a chronic study that evaluated the damaging effects of chronic neuroinflammation and microglial activation after TBI, we demonstrated that delayed administration of CHPG at 1 month after moderate-level controlled cortical impact (CCI) TBI resulted in reduced expression of chronically activated microglia that expressed NADPH oxidase subunits, and these changes were associated with reduced neurodegeneration and improved functional recovery at 4 months post-injury.²⁰

In the current study, we show that acute posttraumatic treatment with the mGluR5 agonist CHPG significantly improves motor and cognitive function recovery and reduces lesion volume after experimental TBI in mice, and that co-administration of an mGluR5 antagonist blocked the protective effects of CHPG. In a separate combined treatment study, co-administration of CHPG with an inhibitor of NADPH oxidase (apocynin) failed to enhance the neuroprotective effects of CHPG alone. Moreover, CHPG treatment reduced NF κ B activity and nitrite production in LPS-stimulated BV2 microglia; knockout of NADPH in microglia cultures (gp91^{phox}^{-/-}) abrogated the protective effects of CHPG treatment in this model. Together, these findings suggest that the beneficial effects of mGluR5 activation after TBI are mediated in part via the inhibition of NADPH oxidase.

Methods

Controlled cortical impact injury

All surgical procedures were performed in accordance with protocols approved by Georgetown University Medical Center

Institutional Animal Care and Use Committee. Our custom-designed CCI injury device²¹ consists of a microprocessor-controlled pneumatic impactor with a 3.5-mm diameter tip. Male C57Bl/6 mice (20–25g) were anesthetized with isoflurane evaporated in a gas mixture containing 70% N₂O and 30% O₂ and administered through a nose mask (induction at 4% and maintenance at 2%). Depth of anesthesia was assessed by monitoring respiration rate and pedal withdrawal reflexes. Mice were placed on a heated pad, and core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and the surgical site was clipped and cleaned with Nolvasan and ethanol scrubs.

A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned to the surface of the exposed dura, and reset to impact the cortical surface. Moderate-level injury was induced using an impactor velocity of 6 m/s and deformation depth of 2 mm as described previously.²² After injury, the incision was closed with interrupted 6-0 silk sutures, anesthesia was terminated, and the animal was placed into a heated cage to maintain normal core temperature for 45 minutes post-injury. All animals were monitored carefully for at least 4 hours after surgery and then daily. Sham animals underwent the same procedure as injured mice except for the impact.

Study 1. At 30 min post-injury, mice received a single intracerebroventricular (ICV) injection of (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG; Tocris Bioscience, Ellisville, MO; *n* = 12) or equal volume vehicle (saline + 1% dimethyl sulfoxide [DMSO]; *n* = 12). A 10-mM solution of CHPG (in saline with 1% DMSO) was injected into the left ventricle (coordinates from bregma = A: -0.5, L: -1.0, V: -2.0) using a 30-gauge needle attached to a Hamilton syringe at a rate of 0.5 μ L/min, with a final volume of 5 μ L, or 50 nmols of CHPG. Another group of mice (*n* = 8) received the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP; Tocris Bioscience; 2 mM solution in saline)²³ by ICV injection immediately before CHPG treatment and served as a negative control group. A sham-injured (craniotomy only) control group was included in the study (*n* = 8). Drug dosages were based on previous studies in spinal cord and focal cerebral ischemia injury models.^{14,15}

Study 2. At 30 min post-injury, mice were randomized into five groups (*n* = 6/group) and administered the following drug treatments: (1) vehicle (saline + 1% DMSO; ICV), (2) CHPG (10 mM; ICV), (3) apocynin (Sigma, St. Louis, MO; 5 mg/kg; IP), (4) CHPG (10 mM; ICV) + apocynin (5 mg/kg; IP), and (5) sham-injured. The dose of apocynin was based on the neuroprotective effects of apocynin treatment in an injury model of global cerebral ischemia.²⁴

Motor function

Fine motor coordination was assessed at 1, 7, 14, and 21 days post-injury using the beam walk task, as previously described.²¹ Briefly, mice were trained for 3 days to cross a narrow wooden beam 6 mm wide and 120 mm in length, which was suspended 300 mm above a 60 mm thick foam rubber pad. The number of foot-faults for the right hindlimb was recorded over 50 steps on each day of testing, and a basal level of competence at this task was established before sham injury or TBI with an acceptance level of < 10 faults per 50 steps.

Cognitive function

The Morris water maze (MWM) was used to evaluate spatial learning deficits in sham-injured and TBI mice as previously

described.²¹ Briefly, sham-injured and TBI mice were trained to locate a hidden submerged platform using extramaze visual information. The apparatus consisted of a large white circular pool (900 mm diameter, 500 mm high, water temperature $24 \pm 1^\circ\text{C}$) with a Plexiglas platform 76 mm diameter painted white and submerged 15 mm below the surface of the water, which had been rendered opaque with the addition of dilute, white non-toxic paint. During training, the platform was hidden in one quadrant (NE position), 14 cm from the side wall.

The mouse was gently placed into the water facing the wall at one of four randomly chosen locations separated by 90 degrees, designated north (N), south (S), east (E), and west (W). The latency to find the hidden platform within a 90 second criterion time was recorded by a blinded observer using a stopwatch. Mice failing to find the platform within the 90 seconds were assisted to it and allowed to remain for 15 seconds on the first trial and 10 seconds on all subsequent trials. There was an interval of 30 minutes, during which time the mice were towel-dried and placed in a heated cage. A series of 16 training trials (4 trials per day over 4 consecutive days; days 14–17 post-injury) were performed starting on day 14 post-injury. To control for visual discriminative ability, the animals were tested in a single trial at least 2 hours after the last training trial to locate a visible flagged platform raised above the water surface.

In vivo MRI

TBI-induced brain lesion volumes was quantified at 21 days post-injury using T2-weighted MRI as previously described ($n=6/\text{group}$).²² Briefly, anesthetized animals were placed in a heated Plexiglas holder, and a respiratory motion detector was positioned over the thorax to facilitate respiratory gating. The Plexiglas holder was then placed in the center of the 7 Tesla magnet bore (Bruker Medical Inc., Billerica, MA) where a 72-mm proton-tuned birdcage coil was positioned. Field homogeneity across the brain was optimized, and a sagittal scout image was acquired (RARE, i.e., rapid acquisition relaxation enhancement pulse sequence), field of vision, $4 \times 4 \text{ cm}$; 128×128 resolution; repetition time (TR) to echo time (TE), 1500/10 millisecond with a RARE factor of 8, making the effective TE 40 millisecond. Multi-slice, multi-echo T2-weighted images were acquired using the following parameters: $3 \times 3 \text{ cm}$ FOV, 256×256 resolution, 1500/10 msec TR:TE, 8 RARE factor, TE 40 msec, $10 \times$ slices, 0.75-mm slice thickness. Lesion volume was quantified from the summation of areas of hyperintensity on each slice, multiplied by slice thickness, for both the ipsilateral and contralateral hemispheres. Contralateral volumes were subtracted from ipsilateral volumes to obtain TBI-induced lesion volumes.

Western immunoblot

At the end of the study (21 days post-injury), vehicle and CHPG treated mice ($n=5/\text{group}$) were anesthetized (100 mg/kg sodium pentobarbital, IP), transcardially perfused with ice-cold saline, and decapitated. A 5-mm area surrounding the lesion epicenter on the ipsilateral cortex was rapidly dissected and immediately frozen on dry ice. Cortical tissue was homogenized in RIPA buffer and centrifuged at 15,000 rpm for 15 minutes at 4°C to isolate proteins, and protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Twenty-five μg of protein was run on SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane using standard techniques. The blots were probed with antibodies against ED1 (1:200, cat. no. MCA1957G, AbD Serotec, Raleigh, NC), and β -actin (1:2000; Sigma-Aldrich, St. Louis, MO) was used as an endogenous control. Immune complexes were detected with the appropriate HRP-conjugated secondary antibodies (KPL, Inc., Gaithersburg, MD) and visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL).

Chemiluminescence was captured on a Kodak Image Station 4000R station (Carestream Health, Rochester, NY) and protein bands were quantified by densitometric analysis using Carestream Molecular Imaging Software. The data presented reflect the intensity of target protein band compared with control and normalized based on the intensity of the endogenous control for each sample (expressed in arbitrary units).

Reactive oxygen species assay

Primary cortical microglia were obtained from postnatal day 2 gp91^{phox+/+} (C57Bl/6) and gp91^{phox/-} mouse pups and cultured as previously described.¹³ Briefly, the whole brain was carefully dissected and homogenized in L15 media (Gibco Invitrogen, Carlsbad, CA). Mixed glial cultures were incubated for 8–10 days at 37°C and 5% CO_2 in Dulbecco Modified Eagle Media (DMEM; Gibco Invitrogen) supplemented with 10% Fetal Calf Serum (Hyclone, Logan, UT), 1% L-glutamine (Gibco Invitrogen), 1% sodium pyruvate (Gibco Invitrogen), and 1% Pen/Strep (Fisher, Pittsburgh, PA). Mixed glial cultures were shaken for 1 h at 100 rpm and 37°C , and detached microglia were collected and seeded at 3×10^5 cells per well in a 96 well plate in DMEM containing 10% Fetal Equine Serum (Hyclone). Intracellular ROS levels were measured using the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Molecular Probes, Eugene, OR). Microglia were pretreated for 1 h with CHPG (4 mM) and stimulated with LPS (100 ng/mL) for 24 h. The cells were incubated with $10 \mu\text{M}$ H_2DCFDA for 45 min at 37°C in 5% CO_2 . Fluorescence was measured using excitation and emission wavelengths of 490 and 535 nm, respectively. Data are presented as percentage of control-treated values. Each treatment group was $n=6$.

NF- κB activity and nitrite assays

BV2 microglia (murine microglial cell line) were grown and maintained in DMEM supplemented with 10% Fetal Bovine Serum (Hyclone) at 37°C and 5% CO_2 . BV2 microglia were seeded at 3×10^5 cells per well in a 96 well plate and were transfected with the 0.15 μg NF- κB -luciferase reporter plasmid (pGL4.32[luc2P/NF- κB /Hygro]; Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. After incubating with DNA-lipofectamine mixtures, the cells were pretreated with CHPG (4 mM) for 1 h and subsequently stimulated with LPS (100 ng/mL) for 8 h. The media were removed and used to measure nitrite release from the activated microglia using a Griess Reagent Assay (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The BV2 microglial cells were washed twice with PBS and lysed with a reporter lysis buffer (Promega). After vortexing and centrifugation at $12,000 \times g$ for 1 min at 4°C , the supernatant was stored -80°C for the luciferase assay. Twenty μL of the BV2 microglial cell extract was mixed with 100 μL of the luciferase assay substrate reagent (Promega) at room temperature, and the luciferase activity was measured using a microplate luminometer (Synergy HT, BioTek Instruments, Winooski, VA). Each treatment group was $n=6$.

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard errors of the mean. Lesion volume and all functional data were performed by an investigator blinded to the treatment group. Functional data for beam walk and acquisition phase of MWM were analyzed by two-way repeated measures analysis of variance (ANOVA) to determine the interactions of post-injury trial and groups, followed by post-hoc adjustments using a Student Newman-Keuls correction. T2-weighted MRI lesion volume, NF- κB activity, nitrite and ROS assays were analyzed by one-way ANOVA, followed by *post-hoc* adjustments using Student Newman-Keuls. Protein expression by

Western immunoblot was analyzed using a Student *t* test. Statistical analysis was performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, San Diego, CA). A $p < 0.05$ was considered statistically significant.

Results

Selective activation of mGluR5 improves functional recovery after TBI

To evaluate the neuroprotective potential of CHPG against TBI-induced functional impairments, we administered CHPG by ICV injection at 30 min post-injury to moderate-level CCI-injured mice and compared them with vehicle (saline)-treated mice. In addition, we administered the mGluR5 antagonist, MPEP, by ICV injection immediately before CHPG, and this combined treatment served as a negative control for the study. Functional assessment of fine motor coordination was assessed on days 1, 7, 14, and 21 post-injury using a well-characterized beam walk test.^{21,22} TBI induced significant sensorimotor impairments at all time points when compared with sham-injured mice ($p < 0.001$ vs. vehicle; Fig. 1A).

CHPG-treated TBI mice had improved sensorimotor performance in this test on days 14 ($p < 0.05$) and 21 ($p < 0.001$) when compared with vehicle-treated TBI mice. In contrast, mice that were administered CHPG + MPEP had similar performance to the vehicle-treated mice, and there was a significant difference between the CHPG-treated and CHPG + MPEP-treated TBI mice at 21 days post-injury ($p < 0.001$). The interaction of the “post-injury day X group” in the beam walk test was statistically significant ($F(9,144) = 2.233$, $p = 0.023$).

Spatial learning was assessed using the acquisition phase of the Morris water maze test.^{21,22} TBI resulted in learning impairments on days 15, 16, and 17 post-injury when compared with sham-injured mice ($p < 0.001$ vs. vehicle; Fig. 1B). CHPG-treated TBI mice showed improvements in spatial learning with significantly reduced latency to find the submerged platform on days 15 ($p < 0.05$), 16 ($p < 0.01$), and 17 ($p < 0.05$) post-injury when compared with vehicle-treated TBI mice. CHPG + MPEP-treated TBI mice had similar latency times as vehicle-treated TBI mice in this test, and there was a significant difference between the CHPG-treated and CHPG + MPEP-treated TBI mice on day 16 post-injury ($p < 0.01$). The interaction of “post-injury day X group” in the Morris water maze test was statistically significant ($F(9,152) = 2.143$, $p = 0.029$).

CHPG treatment results in reduced lesion volumes and microglial activation after TBI

On day 21 post-injury, a randomly selected subgroup of mice ($n = 5$) from the vehicle-, CHPG-, and CHPG + MPEP-treated TBI groups underwent *in vivo* T2-weighted MRI to quantify the lesion size.^{21,22} Representative MRI images from each treatment group are presented in Figure 2A, and arrows indicate regions of TBI-induced cortical and hippocampal damage (hyperintense signal) on day 21 post-injury. TBI resulted in a large lesion in vehicle-treated mice ($0.0307 \pm 0.0076 \text{ cm}^3$; Fig. 2B), and the TBI-induced lesion was significantly reduced in CHPG-treated TBI mice ($0.0128 \pm 0.0010 \text{ cm}^3$).

In contrast, mice that received CHPG and MPEP had similar lesions ($0.02184 \pm 0.0027 \text{ cm}^3$) to the vehicle-treated TBI mice. After T2-weighted MRI imaging, the mice were euthanized, and cortical tissue surrounding the lesion site was isolated and proteins were extracted for Western immunoblot analysis. Western blots for

FIG. 1. mGluR5 activation by (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) treatment improves functional recovery after traumatic brain injury (TBI). **(A)** Fine motor coordination deficits were quantified using the beam walk test. The interaction of “post-injury day X group” was statistically significant ($F(9,144) = 2.233$, $p = 0.0023$), and TBI induced significant sensorimotor impairments at all time points when compared with sham-injured mice ($***p < 0.001$ vs. vehicle). Central administration of CHPG at 30 min post-injury improved fine motor coordination at 14 and 21 days post-injury ($^+p < 0.05$ and $^{+++}p < 0.001$ vs. vehicle respectively). In contrast, administration of the mGluR5 antagonist, MPEP, immediately before CHPG reversed the improvement in sensorimotor function and resulted in a significant difference between the CHPG-treated and CHPG + MPEP-treated TBI groups at 21 days post-injury ($^{^^}p < 0.001$ vs. CHPG + MPEP). **(B)** Spatial learning was assessed using the acquisition phase of the Morris water maze test. The interaction of “post-injury day X group” was statistically significant ($F(9,152) = 2.143$, $p = 0.029$), and TBI resulted in learning impairments on days 15, 16, and 17 post-injury when compared with sham-injured mice ($***p < 0.001$ vs. vehicle). CHPG-treated TBI mice showed improvements in spatial learning with significantly reduced latency to find the submerged platform on days 15 ($^+p < 0.05$), 16 ($^{++}p < 0.01$), and 17 ($^+p < 0.05$) post-injury when compared with vehicle-treated TBI mice. CHPG + MPEP-treated TBI mice had similar latency times as vehicle-treated TBI mice, and there was a significant difference between the CHPG-treated and CHPG + MPEP-treated TBI groups on day 16 post-injury ($^{^^}p < 0.01$ vs. CHPG + MPEP). Analysis by two-way repeated measures analysis of variance, followed by *post-hoc* adjustments using a Student Newman-Keuls correction in (A) and (B). Mean $\pm$ standard error of the mean ($n = 12$ for vehicle, CHPG TBI mice, $n = 8$ for CHPG + MPEP TBI mice and sham-injured mice).

ED1, a well known marker for activated microglia, were performed on vehicle- and CHPG-treated TBI tissue (Fig. 2C); CHPG treatment significantly reduced ED1 protein expression at 21 days post-injury ($p < 0.05$ vs. vehicle) indicating reduced microglial activation in the CHPG-treated group.

FIG. 2. CHPG treatment reduces traumatic brain injury (TBI)-induced lesion volume and microglial activation at 21 days post-injury. (A) T2-weighted MRI was performed on a subset of vehicle-, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG)- and CHPG + MPEP-treated TBI mice at 21 days post-injury, and representative images of each group are shown. (B) Analysis of lesion volumes demonstrated that CHPG-treated TBI mice had significantly reduced lesion size ($*p < 0.05$) when compared with vehicle-treated TBI mice. (C) Western immunoblot analysis for ED-1, a marker of activated microglia, demonstrate that CHPG-treated TBI mice had reduced ED-1 protein expression ($*p < 0.05$) when compared with vehicle-treated TBI mice. Analysis by one-way analysis of variance, followed by *post-hoc* adjustments using a Student Newman-Keuls correction in (B), Student *t* test in (B). Mean $\pm$ standard error of the mean ($n = 6$ /group).

Attenuation of microglial activation by mGluR5 stimulation is mediated by the inhibition of NADPH oxidase

Previously, we demonstrated that selective activation of microglial mGluR5 receptors resulted in attenuated microglial activation and reduced microglial-mediated neurotoxicity in neuron-microglia co-culture assays.^{12,13} Here, we extended those studies and evaluated the effect of CHPG treatment on NF κ B signaling in a model of LPS-stimulated BV2 microglial cell activation using a

luciferase reporter assay. LPS resulted in a significant increase in NF κ B activity in BV2 microglia (Fig. 3A; $p < 0.001$ vs. control), and CHPG treatment (4 mM) significantly reduced NF κ B activity ($p < 0.001$ vs. LPS). CHPG treatment alone did not induce NF κ B activity in this assay. In addition, nitrite levels were measured in the media from the BV2 microglia transfected with the NF κ B-luciferase reporter plasmids. There was a significant increase in nitrite levels in LPS-stimulated BV2 microglia at 8 hours after stimulation (Fig. 3B; $p < 0.001$ vs. control). CHPG treatment significantly reduced the nitrite levels ($p < 0.001$ vs. LPS), whereas CHPG

FIG. 3. mGluR5 activation attenuates NFκB signaling in microglia and the anti-inflammatory effects of CHPG treatment are mediated by the inhibition of NADPH oxidase. (A) Lipopolysaccharide (LPS) stimulation resulted in a significant increase in NFκB activity in BV2 microglia (** $p < 0.001$ vs. control), and (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) treatment significantly reduced NFκB activity (** $p < 0.001$ vs. LPS) in a NF-κB-luciferase reporter assay. (B) Nitrite levels were measured in the media from the BV2 microglia transfected with the NF-κB-luciferase reporter plasmids. LPS stimulation resulted in a significant increase in nitrite levels (** $p < 0.001$ vs. control), and CHPG treatment significantly reduced LPS-stimulated nitrite production (** $p < 0.001$ vs. LPS). (C) LPS stimulation resulted in a significant increase in intracellular reactive oxygen species (ROS) levels in gp91^{phox+/+} microglia (** $p < 0.001$ vs. control [gp91^{phox+/+}]). CHPG treatment significantly reduced ROS levels in gp91^{phox+/+} microglia (** $p < 0.001$ vs. LPS [gp91^{phox+/+}]). In gp91^{phox-/-} microglia, LPS also resulted in a significant increase in intracellular ROS levels (### $p < 0.001$ vs. control [gp91^{phox-/-}]). These levels, however, were significantly reduced when compared with those in gp91^{phox+/+} microglia (^^ $p < 0.001$ vs. LPS [gp91^{phox+/+}]). Notably, CHPG treatment failed to significantly reduce intracellular ROS levels in gp91^{phox-/-} microglia. Analysis by one-way analysis of variance, followed by *post-hoc* adjustments using a Student Newman-Keuls correction. Mean $\pm$ standard error of the mean ($n = 6$ /treatment).

treatment alone did not produce nitrite in the absence of LPS stimulation.

Previously, we also demonstrated that CHPG treatment reduced NADPH oxidase activity in LPS-stimulated BV2 microglia and that siRNAs knockdown of either of the two membrane subunits of NADPH oxidase (p22^{phox} or gp91^{phox}) resulted in a loss of the protective actions of CHPG treatment.¹³ Here, we extended those studies and measured ROS production in LPS-stimulated primary microglia from gp91^{phox+/+} and gp91^{phox-/-} mice. LPS stimulation resulted in a significant increase in intracellular ROS levels in gp91^{phox+/+} microglia (Fig. 3C; $p < 0.001$ vs. control [gp91^{phox+/+}]); CHPG treatment (4 mM) significantly reduced ROS levels in gp91^{phox+/+} microglia ($p < 0.001$ vs. LPS [gp91^{phox+/+}]). LPS also resulted in a significant increase in intracellular ROS levels in gp91^{phox-/-} microglia ($p < 0.001$ vs. control [gp91^{phox-/-}]); however, these levels were significantly reduced when compared with those in gp91^{phox+/+} microglia ($p < 0.001$ vs. LPS [gp91^{phox+/+}]). CHPG pre-treatment failed to significantly reduce intracellular

ROS levels in gp91^{phox-/-} microglia, indicating that the protective effects of CHPG are mediated by the inhibition of NADPH oxidase in microglia.

Inhibition of NADPH oxidase does not enhance the neuroprotective effects of CHPG after TBI

Inhibition of NADPH oxidase has been shown to be neuroprotective in acute brain injury models such as ischemia-reperfusion injury and TBI.^{25–28} Based on our *in vitro* studies and our previous studies *in vivo*, which demonstrated that CHPG treatment reduced NADPH oxidase expression in injured brain and spinal cord tissue, we wanted to determine if the neuroprotective effects of CHPG treatment after TBI are because of the inhibition of NADPH oxidase, or if these two neuroprotective mechanisms operate independently. We designed a combination treatment study to test this hypothesis, and after TBI mice were randomized into one of five treatment groups and were administered the following drug

treatments at 30 min post-injury: (1) vehicle (saline; ICV), (2) CHPG (ICV), (3) apocynin (IP), (4) CHPG (ICV) + apocynin (IP), and (5) sham-injury; and evaluated by functional testing and lesion volume analysis.

In the beam walk test, TBI induced significant sensorimotor impairments at all time points when compared with sham-injured mice ($p < 0.001$ vs. vehicle; Fig. 4A). Similar to our first study, CHPG-treated TBI mice had improved sensorimotor performance in this test and had a significantly reduced number of footfaults on days 7 ($p < 0.01$), 14 ($p < 0.05$), and 21 ($p < 0.001$) post-injury when compared with vehicle-treated TBI mice. Apocynin-treated TBI mice also had significantly improved sensorimotor performance on days 14 ($p < 0.05$) and 21 ($p < 0.05$) when compared with vehicle-treated TBI mice. TBI mice that received the combination treatment of CHPG + apocynin had significantly reduced number of footfaults on days 7 ($p < 0.05$), 14 ($p < 0.05$), and 21 ($p < 0.001$) post-injury. Notably, the combined treatment (CHPG + apocynin) did not further enhance fine motor coordination performance when compared with CHPG-treated TBI mice. The interaction of “post-injury day X group” was statistically significant ($F(12,100) = 2.156$, $p = 0.020$) in this test.

In the Morris water maze test, TBI induced significant spatial learning impairments at all time points when compared with sham-injured mice ($p < 0.05$, $p < 0.01$ vs. vehicle; Fig. 4B). CHPG-treated TBI mice showed improvements in spatial learning with significantly reduced latency to find the submerged platform on day 17 ($p < 0.01$) post-injury when compared with vehicle-treated TBI mice. In addition, there were trends toward improvements in spatial learning in the other treatment groups; apocynin and CHPG + apocynin treatment resulted in non-significant reductions in latency to reach the submerged platform when compared with the vehicle-treated TBI mice (latency on day 17: vehicle = 60 ± 4 ; apocynin = 40 ± 10 ; CHPG + apocynin = 45 ± 7 sec). The interaction of “post-injury day X group” did not reach statistical significance ($F(12,100) = 0.814$, $p = 0.636$) in this test.

Finally, we evaluated TBI-induced lesion volumes at 21 days post-injury in each of the treatment groups (Fig. 5A), and there were significantly reduced lesion volumes in the CHPG-treated ($0.0242 \pm 0.0086 \text{ cm}^3$), apocynin-treated ($0.0234 \pm 0.0044 \text{ cm}^3$), and CHPG + apocynin-treated ($0.0203 \pm 0.0056 \text{ cm}^3$) groups when compared with the lesion of the vehicle-treated group ($0.0470 \pm 0.0068 \text{ cm}^3$; $p < 0.05$ for each treatment vs. vehicle). There was no significant difference between each of the treatments, however, and no additive effect of CHPG with apocynin on TBI-induced lesion volumes. Representative MRI images for each treatment group are shown in Figure 5B. Therefore, this combined treatment study in which coadministration of CHPG with an inhibitor of NADPH oxidase failed to enhance the neuroprotective potential of CHPG alone suggests that the beneficial effects of activating mGluR5 after TBI are mediated, in part, via the inhibition of NADPH oxidase.

Discussion

Although most studies of mGluRs have focused on their effects on neuronal function and modulation, mGluR5 receptors are also expressed by glial cells, including astrocytes, microglia, and oligodendrocytes.^{5–7} Thus, the protective actions of selective mGluR5 activation may reflect effects on different cell types. Activation of mGluR5 significantly inhibits apoptosis in models of A β and MPP+ induced-neuronal cell death,^{8–11} and provides neuroprotection in response to mechanical injury of cultured neurons via

FIG. 4. Inhibition of NADPH oxidase does not enhance (RS)-2-chloro-5-hydroxyphenylglycine (CHPG)-mediated functional recovery after TBI. (A) Fine motor coordination deficits were quantified using the beam walk test. The interaction of “post-injury day X group” was statistically significant ($F(12,100) = 2.156$, $p = 0.020$), and TBI induced significant sensorimotor impairments at all time points when compared with sham-injured mice ($***p < 0.001$ vs. vehicle). CHPG-treated TBI mice had improved sensorimotor performance and had significantly reduced number of footfaults on days 7, 14, and 21 post-injury ($^{++}p < 0.01$, $^{+}p < 0.05$ and $^{+++}p < 0.001$, respectively) when compared with vehicle-treated TBI mice. Apocynin-treated TBI mice had significantly improved sensorimotor performance on days 14 and 21 post-injury ($^{*}p < 0.05$ vs. vehicle), and TBI mice that received the combination treatment of CHPG + apocynin had significantly reduced numbers of footfaults on days 7, 14, and 21 post-injury ($^{*}p < 0.05$, and $^{###}p < 0.001$) when compared with vehicle-treated TBI mice. The combined treatment (CHPG + apocynin) did not further enhance fine motor coordination performance when compared with CHPG-treated TBI mice. (B) Analysis of spatial learning in TBI mice was assessed by the Morris water maze test and the interaction of “post-injury day X group” did not reach statistical significance ($F(12,100) = 0.814$, $p = 0.636$). There were significant TBI-induced learning impairments, however, on days 15 ($^{*}p < 0.05$), 16 ($^{*}p < 0.05$), and 17 ($^{**}p < 0.01$) post-injury when compared with sham-injured mice. Each treatment resulted in trends toward reduced latency to reach the submerged platform when compared with the vehicle-treated TBI mice, with CHPG treatment resulting in a significant reduction on day 17 post-injury ($^{++}p < 0.01$ vs. vehicle). Analysis by two-way repeated measures analysis of variance, followed by *post-hoc* adjustments using a Student Newman-Keuls in (A) and (B). Mean $\pm$ standard error of the mean ($n = 6/\text{group}$).

PKC-dependent activation of the MEK/ERK signaling pathway.²⁹ Furthermore, in cocultures of neurons and astrocytes, administration of CHPG significantly reduced NMDA-mediated currents after stretch-injury³⁰; however, in the absence of astrocytes, CHPG did not modulate stretch-injury induced NMDA responses, thereby highlighting the critical role of astrocytes in this injury response. In

FIG. 5. Inhibition of NADPH oxidase does not enhance the neuroprotective effects of (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) after traumatic brain injury (TBI). (A) Analysis of T2-weighted MRI lesion volumes demonstrated that CHPG-, apocynin-, and CHPG+apocynin-treated TBI mice had significantly reduced lesions ($*p < 0.05$) when compared with vehicle-treated TBI mice. (B) Representative T2-weighted MRI images for each group are shown. Analysis by one-way analysis of variance, followed by *post-hoc* adjustments using a Student Newman-Keuls correction in (A). Mean $\pm$ standard error of the mean ($n = 6/\text{group}$).

addition, mGluR5 is expressed on microglia, and selective activation of mGluR5 is anti-inflammatory in LPS- and IFN γ -induced models of microglial activation.^{12,13} Therefore, pleiotropic neuroprotective effects of selective mGluR5 activation in neurons and glia likely contribute to the considerable neuroprotective properties of CHPG in experimental models of CNS injury.

The present studies and previously published reports from our laboratory demonstrate that selective activation of mGluR5 by CHPG has powerful neuroprotective effects *in vivo*. For example, in an experimental model of moderately severe spinal cord contusion injury, we demonstrated that CHPG treatment for 7 days resulted in improved motor function recovery, reduced lesion volume, as well as sparing of white matter through 28 days post-injury.¹⁵ More recently, we showed that delayed administration of CHPG at 1 month after moderate-level TBI arrested the expansion of the TBI-induced lesion over time, reduced white matter loss and

hippocampal neurodegeneration, and improved functional recovery at 4 months post-injury.²⁰ In both *in vivo* studies, CHPG treatment reduced chronic microglial activation and the expression of NADPH oxidase after injury.

Our current data are consistent with these findings and a recent report showing that treatment with CHPG before TBI in rats resulted in reduced lesion size and neuronal apoptosis in the injured cortex.³¹ We showed that central administration of CHPG at 30 min post-injury resulted in significantly improved sensorimotor and cognitive function recovery and reduced TBI-induced lesion volumes at 21 days post-injury. Further, we included a negative control group in these behavioral studies in which mice were coadministered the mGluR5 antagonist MPEP with CHPG, and these animals performed similarly to vehicle-treated animals in all outcome measures. Because MPEP blocked the protective effects of CHPG treatment in this control group, these data suggest that the

therapeutic actions of CHPG treatment after TBI were mediated by the selective activation of mGluR5 receptors.

In experimental models of TBI, SCI, or excitotoxic (ibotenic acid) lesions to the brain, mGluR5 expression has been shown to be up-regulated in microglia that surround the lesion site.^{15,20,32,33} We have also demonstrated mGluR5 protein expression in cultured primary microglia and a microglial cell line (BV2 cells),^{12,13} and that selective activation of mGluR5 by CHPG or DHPG/CPCCOEt (group I mGluR agonist and mGluR1 receptor antagonist combination) attenuated microglial activation induced by LPS or IFN γ ,^{5,12,13,34} as well as reduced the levels of proinflammatory mediators such as TNF α , iNOS, NO, and ROS. Moreover, selective activation of mGluR5 on microglia reduced NADPH oxidase enzymatic activity and significantly attenuated microglial-mediated neurotoxicity in a coculture model.¹³

The protective effects of CHPG treatment in microglia were abrogated by selective knockdown of NADPH oxidase by siRNAs directed against either of the two membrane subunits of enzyme complex (gp91^{phox} or p22^{phox}). In this study, we used primary microglia from gp91^{phox}+/+ and gp91^{phox}-/- mice and demonstrated in a model of LPS-stimulated microglial activation that CHPG treatment significantly reduced ROS production in gp91^{phox}+/+ microglia, but failed to reduce LPS-induced ROS production in gp91^{phox}-/- microglia. These data support our previous siRNA knockdown of NADPH oxidase subunits in BV2 microglia¹³ and suggest that the anti-inflammatory effects of CHPG are mediated in part via the inhibition of NADPH oxidase.

In addition, we also provide new data on the effects of mGluR5 activation on downstream proinflammatory signaling cascades in microglia. It is well established that LPS binds to Toll-like receptor-4 (TLR-4) on microglia and thus initiates MAP kinase and NF κ B signaling pathways, which ultimately lead to increased transcriptional activity of proinflammatory genes (e.g., iNOS).^{35,36} NADPH oxidase-derived ROS has also been demonstrated to play a critical role in the induction of these signaling cascades in microglia, and inhibition of NADPH oxidase by diphenylene iodonium chloride reduces NF κ B activity and proinflammatory gene expression.³⁶ In the present study we show that CHPG treatment reduces LPS-induced ROS production in microglia, and it also significantly attenuates NF κ B activity and the subsequent release of nitric oxide from BV2 microglia after LPS stimulation. These findings confirm the potent anti-inflammatory effects of mGluR5 activation in microglia and demonstrate that CHPG treatment inhibits a key proinflammatory signaling pathway in microglia.

To further address whether CHPG protection is mediated by inhibition of NADPH oxidase, or if the two systems are operating separately, we performed a TBI study using a combination treatment approach in which vehicle, CHPG, apocynin, or CHPG + apocynin were administered at 30 min post-injury. The study revealed that the CHPG- and CHPG + apocynin-treated groups resulted in the best improvements in sensorimotor function recovery and equivalent reductions in post-traumatic lesion volumes; however, there was no significant difference between CHPG and CHPG + apocynin groups—i.e., no additive effect of CHPG with apocynin. These data suggest that both drugs target the same pathways, and therefore the neuroprotective effects of selective activation of mGluR5 receptors by CHPG treatment may be in part because of the direct inhibition of NADPH oxidase.

NADPH oxidase has been implicated as a common and essential mechanism of microglia-mediated neurotoxicity in numerous neurodegenerative diseases, such as Parkinson disease and Alzheimer disease.¹⁷ Further, we recently demonstrated that NADPH

oxidase plays a key role in microglial activation after TBI and that gp91^{phox} is highly expressed in chronically activated microglia up to 4 months post-injury. Inhibition of NADPH oxidase by apocynin treatment or gp91^{phox}-/- is neuroprotective after ischemia-reperfusion injury and results in reduced injury-induced oxidative damage and lipid degradation and reduced microglial activation.^{25,27} Apocynin treatment also reduces neuronal loss and the size of the lesion in this model. Our study is consistent with these reports because apocynin treatment significantly reduced the TBI-induced lesion volume by approximately 50% and resulted in trends toward improved motor and cognitive function recovery. Another study has also demonstrated the neuroprotective effects of NADPH oxidase inhibition by apocynin after TBI and showed reduced oxidative damage, neurodegeneration, and microglial activation in apocynin-treated TBI mice.²⁸

Conclusion

Our data demonstrate that activation of mGluR5 using the selective agonist, CHPG, at 30 min after moderate-level TBI in mice results in significantly improved sensorimotor and cognitive function recovery and reduced TBI-induced lesion volumes. The therapeutic actions were mediated by mGluR5, because coadministration of an mGluR5 antagonist MPEP blocked the protective effects of CHPG on these outcomes. We also demonstrated that mGluR5 stimulation significantly reduces NF κ B signaling in microglial cultures and that the anti-inflammatory effects of CHPG treatment were abrogated in microglial cultures prepared from gp91^{phox}-/- mice. Further, combined treatment TBI studies in which the NADPH oxidase inhibitor apocynin was coadministered with CHPG failed to enhance functional recovery compared with CHPG treatment alone, thereby suggesting that the beneficial effects of mGluR5 stimulation are likely mediated through the inhibition of NADPH oxidase.

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Author Disclosure Statement

KRB, DJL, and AIF are listed as inventors for a use patent from Georgetown University for mGluR5 agonists in the treatment of neuroinflammation. For the remaining authors, no competing financial interests exist.

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