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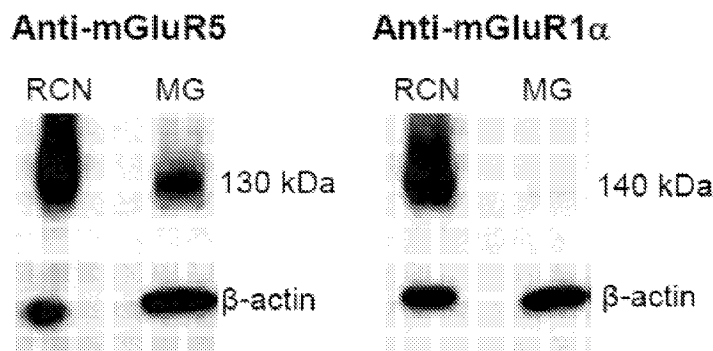


FIGURE 1

(57) Abstract: Methods for the treatment of neuroinflammation or neurotoxicity are provided. For example, a method is provided for preventing neuroinflammation or neurotoxicity in a subject comprising administering to a subject an mGluR5 agonist. Also provided is a method of preventing neuroinflammation or neurotoxicity in a subject, wherein the subject has a central nervous system injury, comprising administering to the subject an mGluR5 agonist. Methods can further comprise preventing neuroinflammation or neurotoxicity in a subject, wherein the subject has or is suspected of having a neurodegenerative disease. The provided methods are useful for treating acute or chronic injury or disorder. For example, provided are methods of treating a subject having a nervous system disease or injury, comprising administering to the subject having the nervous system disease or injury an mGluR5 agonist.

REDUCING OR PREVENTING

NEUROINFLAMMATION OR NEUROTOXICITY

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No.
5 61/097,155, filed September 15, 2008, which is incorporated by reference in its
entirety as part of this application.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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10 has certain rights in this invention.

BACKGROUND

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors
that modulate glutamatergic activity. Within the central nervous system, these
receptors have been studied largely in neurons, although they are also found in glia.
15 Classified into three groups based upon their signal transduction pathways and
pharmacological profiles, activation of several mGluR subtypes affect neuronal
viability both *in vitro* and after central nervous system injury.

SUMMARY

Methods for the treatment of neuroinflammation or neurotoxicity are provided.
20 For example, a method is provided for preventing neuroinflammation or neurotoxicity
in a subject comprising administering to a subject an mGluR5 agonist. The agonist
can be administered by a variety of methods, including, for example, systemically,
intraventricularly, or directly into the central nervous system of the subject (e.g., at a
local site of injury or surgery). The mGluR5 agonist can, for example, be selected
25 from the group consisting of a small molecule, an inhibitory RNA, or a polypeptide.

Also provided is a method of preventing neuroinflammation or neurotoxicity
in a subject, wherein the subject has a central nervous system injury, comprising
administering to the subject an mGluR5 agonist. A central nervous system injury can,
for example, be a spinal cord injury, a head injury, or an injury resulting from a stroke.

Methods can further comprise preventing neuroinflammation or neurotoxicity in a subject, wherein the subject has or is suspected of having a neurodegenerative disease. A neurodegenerative disease includes, for example, Alzheimer's Disease, Parkinson's Disease, and multiple sclerosis.

5 The methods are useful for treating acute or chronic injury or disorder. For example, provided are methods of treating a subject having a nervous system disease or injury, comprising administering to the subject having the nervous system disease or injury an mGluR5 agonist. The nervous system injury is optionally selected from the group consisting of a stroke, traumatic brain injury, and spinal cord injury. The
10 nervous system disease is optionally selected from the group consisting of Alzheimer's Disease, Parkinson's Disease and multiple sclerosis.

Also provided is a method of reducing activation of a microglial cell comprising contacting the microglia cell with an mGluR5 agonist. The method optionally comprises contacting the microglia cell *in vitro* or *in vivo*.

15 The details are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the methods and compositions will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a Western blot of rat microglia cultures demonstrating mGluR5
20 protein expression but low mGluR1 α expression.

Figures 2A and 2B are bar graphs demonstrating that ROS production (Fig. 2A) and NO production (Fig. 2B) were inhibited by CHPG treatment prior to LPS stimulation. Bars represent mean $\pm$ SEM; * p <0.05 vs. control; ** p <0.001 vs. control; # p <0.05 vs. LPS; ## p <0.001 vs. LPS.

25 Figures 3A-C are bar graphs demonstrating that proliferation (Fig. 3A), NO production (Fig. 3B) and TNF α production (Fig. 3C) are inhibited by CHPG treatment prior to LPS stimulation. All measurements were assessed from 30 minutes to 24 hours. Bars represent mean $\pm$ SEM; * p <0.05 vs. control; ** p <0.001 vs. control; # p <0.05 vs. LPS; ## p <0.001 vs. LPS.

30 Figure 4 is a bar graph demonstrating that CHPG inhibits microglial-induced neurotoxicity. Bars represent mean $\pm$ SEM; * p <0.05 vs. control; # p <0.05 vs. LPS; + p <0.05 vs. CHPG + LPS.

Figures 5A-F are bar graphs from microglia cultures from mGluR5 wild-type (+/+), Figs. 5A, C, E) or knockout (-/-, Figs. 5B, D, F) mice. LPS stimulation resulted in an increase in microglial proliferation (Figs. 5A, B), NO release (Figs. 5C, D) and ROS production (Figs. 5E, F) that was attenuated by CHPG in wild-type microglia (Figs. 5A, C, E). However, proliferation and NO production were not attenuated in knockout microglia (Figs. 5B, D, F). Bars represent mean $\pm$ SEM; * p <0.05 vs. control; ** p <0.001 vs. control; # p <0.05 vs. LPS; NS: not significant vs. LPS.

Figures 6 A-D are bar graphs demonstrating cAMP expression was increased by CHPG treatment, in comparison to control treatment (Fig. 6A); NO production was reduced when pretreated with cAMP inducers forskolin (50 μ M) and dbcAMP (200 μ M) on LPS-stimulated microglia (Fig. 6B); blocking cAMP production with the adenylyl cyclase inhibitor SQ22356 (0.1 - 500 μ M; Fig. 6C) or rpcAMPs (10 - 500 μ M, Fig. 6D) did not have any significant effect on the reduction in ROS production induced by CHPG. Bars represent mean $\pm$ SEM; * p <0.05 vs. control; ** p <0.001 vs. control; # p <0.05 vs. LPS.

Figures 7 A-D are bar graphs showing that transduction pathways involved in CHPG signalling in microglia indicate the involvement of PLC, PKC and calcium. Fig. 7A is a bar graph demonstrating that PI hydrolysis was increased when microglia were treated with CHPG. Fig. 7B is a bar graph demonstrating that pre-treatment of microglial cultures with the PLC β inhibitor U-73122 blocked the ability of CHPG to reduce LPS-induced ROS production at 10 μ M. Fig. 7C is a bar graph demonstrating that CHPG-induced reduction of proliferation was also blocked by the calcium chelator BAPTA-AM and the PKC inhibitor R0318220 (10 μ M and 1.5 μ M, respectively). Fig. 7D is a bar graph demonstrating that R0318220 and BAPTA-AM also inhibited CHPG's effects on NO production. Bars represent mean $\pm$ SEM; * p <0.05 vs. control; ** p <0.001 vs. control; # p <0.05 vs. LPS; + p <0.05 vs. CHPG + LPS; ++ p <0.001 vs. CHPG + LPS.

Figure 8A is a Western immunoblot confirming mGluR5 expression in cultured BV2 and primary cortical microglia whereas mGluR1 α expression was weak and negligible by comparison. Rat cortical neuron (RCN) samples were run alongside as a positive control for mGluR1 α .

Figure 8B is a graph showing that the selective mGluR5 agonist, CHPG, stimulated significant phosphoinositide hydrolysis in BV2 microglia (* p <0.05, versus

control; ANOVA), thereby demonstrating the presence of a functional mGluR5 receptor. Values represent means $\pm$ SEM from at least 5 independent measurements.

Figures 9A-D are graphs showing selective stimulation of mGluR5 attenuates microglial activation. Fig. 9A shows CHPG dose-dependently attenuated LPS-stimulated nitric oxide (NO) production in BV2 microglia. Fig. 9B shows the group I mGluR agonist, DHPG (50 μ M), when applied to microglia in combination with the mGluR1 antagonist, CPCCOEt (100 μ M), significantly attenuated LPS-stimulated NO production, whereas DHPG in combination with the mGluR5 agonist, MTEP (10 μ M), failed to modulate LPS-stimulated NO production. Fig. 9C shows the mGluR5 antagonist, MTEP (10 μ M), reversed CHPG's attenuation of LPS-stimulated NO production. Fig. 9D shows CHPG's attenuation of LPS-stimulated TNF α production was significantly reduced in mGluR5 siRNA transfected BV2 microglia when compared to control siRNA transfected cells. mGluR5 receptor knockdown was confirmed by Western immunoblotting. For each of the above, BV2 microglia were pre-treated with various concentrations of drug for 1 hr and stimulated with LPS (100ng/ml) for 24 hrs. Values represent means $\pm$ SEM from at least 6 independent measurements. ***p<0.001, versus control; +p<0.05, +++p<0.001 versus LPS; ###p<0.001 versus LPS+DHPG+CPCCOEt; ^^p<0.001 versus LPS+CHPG; ANOVA.

Figures 10A-D are Western immunoblots and graphs showing that mGluR5 activation attenuates the release of LPS-stimulated pro-inflammatory mediators and microglial-mediated neurotoxicity. LPS-stimulation (100ng/ml) in BV2 microglia significantly increased iNOS expression at 4, 15 and 24 hrs (Fig. 10Ai), NO production at 15 and 24 hrs (Fig. 10B), and TNF α production at 1, 4, 15, and 24 hrs (Fig. 10C) (***p<0.001, versus control; ANOVA). Pre-treatment of cells with CHPG (4mM) significantly attenuated LPS-stimulated increases in each measure at the times indicated (++p<0.01, +++p<0.001, versus LPS; ANOVA). LPS-stimulation also increased iNOS expression in primary cortical microglia (Fig. 10Aii, ***p<0.001, versus control; ANOVA) and CHPG pre-treatment significantly reduced iNOS expression after 24 hrs (+++p<0.001, versus LPS; ANOVA). Conditioned media from LPS-stimulated microglia induced B35 neuroblastoma cell death (***p<0.001, versus control; ANOVA). However, pre-treatment of microglia with CHPG prior to LPS-stimulation and addition of conditioned media to neurons resulted in reduced cell

death ($^{+++}p < 0.001$, versus LPS; ANOVA). Values represent means $\pm$ SEM from at least 6 independent measurements. Representative iNOS immunoblots are shown in Fig. 10Ai and Fig. 10Aii.

Figure 11 is a Western immunoblot and graph confirming that CHPG pre-treatment significantly attenuated p22^{PHOX} and gp91^{PHOX} protein expression following LPS-stimulation in BV2 microglia ($^{***}p < 0.001$, versus control; $^{+}p < 0.05$, versus LPS; ANOVA). Values represent means $\pm$ SEM from at least 6 independent measurements. Representative p22^{PHOX} and gp91^{PHOX} immunoblots are shown.

Figures 12A and B are graphs showing mGluR5 activation inhibits microglial NADPH oxidase activity and intracellular ROS generation. Fig. 12A shows LPS-stimulation of BV2 microglia resulted in increased microglial NADPH oxidase enzymatic activity after 4 hrs ($^{***}p < 0.001$, LPS versus control; ANOVA). Pre-treatment of microglia with CHPG or the NADPH oxidase inhibitor, apocynin (1mM), significantly reduced LPS-stimulated NADPH oxidase activity ($^{+++}p < 0.001$, versus LPS; ANOVA). Fig. 12B shows LPS-stimulation caused significant intracellular ROS generation in BV2 microglia after 24 hrs ($^{***}p < 0.001$, versus control; ANOVA). Pre-treatment with CHPG or apocynin significantly reduced LPS-stimulated ROS production ($^{+}p < 0.05$ and $^{++}p < 0.01$, versus LPS; ANOVA) at this time while neither drug had any effect on its own. Values represent means $\pm$ SEM from at least 6 independent measurements.

Figures 13A-C are Western immunoblots and graphs showing that siRNA knockdown of p22^{PHOX} and gp91^{PHOX} reduces the protective effects of mGluR5 activation. Fig. 13A shows that Western immunoblotting demonstrated a 52% reduction in p22^{PHOX} and 60% reduction in gp91^{PHOX} protein expression when BV2 microglia were transfected with p22^{PHOX}-siRNA (Fig. 13Ai) and gp91^{PHOX}-siRNA (Fig. 13Aii) as compared to scrambled control-siRNA transfected cells. Representative immunoblots are shown of 6 independent measurements. Fig. 13B shows CHPG's attenuation of LPS-stimulated NO production was significantly reduced in p22^{PHOX}- and gp91^{PHOX}-siRNA transfected BV2 microglia when compared to control-siRNA transfected cells ($^{***}p < 0.001$, versus control; ANOVA). Fig. 13C shows CHPG's attenuation of LPS-stimulated TNF α release was significantly reduced in p22^{PHOX}- and gp91^{PHOX}-siRNA transfected BV2 microglia when compared

to control-siRNA transfected cells (** $p < 0.001$, versus control; ANOVA). Values represent means $\pm$ SEM from at least 6 independent measurements.

Figures 14A and B are graphs showing that mGluR5 activation attenuates IFN γ -stimulated microglial activation and the release of pro-inflammatory mediators.

5 BV2 microglia were pre-treated with CHPG (4mM) for 1 hr and stimulated with increasing concentrations of IFN γ (0.5 and 2ng/ml) for a further 24 hrs. Fig 14A shows IFN γ dose-dependently stimulated the production of NO in microglia (** $p < 0.001$ versus control; ANOVA), while CHPG pre-treatment significantly attenuated IFN γ -stimulated NO production (++ $p < 0.001$ versus IFN γ ; ANOVA).

10 Fig. 14B shows that IFN γ dose-dependently stimulated the release of increasing concentrations of TNF α from microglia (** $p < 0.001$ versus control; ANOVA), whereas CHPG pre-treatment significantly attenuated IFN γ -stimulated TNF α release (++ $p < 0.001$ versus IFN γ ; ANOVA). Values represent means $\pm$ SEM from at least 6 independent measurements.

15 Figures 15A and B are graphs showing functional effects of mGluR5 activation following traumatic spinal cord injury. Hindlimb locomotor function was assessed using the BBB score at days 1, 7, 14, 21 and 28 after injury (Fig. 15A). CHPG treatment resulted in a significant improvement in BBB score by day 14 post-injury, which continued through day 28. Squares = Vehicle, triangles = CHPG. The
20 slope of the line was also assessed (Fig. 15B). CHPG treatment resulted in a significantly greater rate of recovery than vehicle. Bars represent mean $\pm$ SEM; * $p < 0.05$.

Figures 16A-C show MRI-based lesion volume measurements following traumatic spinal cord injury. At 28 days post-injury, rats underwent T2-weighted
25 MRI imaging. Hyperintense regions (arrows) indicate lesion sites in vehicle treated (Fig 16A) and CHPG treated (Fig. 16B) animals. The MRI-based lesion volume of these hyperintense regions was assessed and demonstrated a significant reduction with CHPG treatment (Fig. 16C). Bars represent mean $\pm$ SEM; * $p < 0.05$.

Figures 17A-C show histological effects of mGluR5 activation following
30 traumatic spinal cord injury. Using eriochrome stained tissue slides (Fig. 17A), cavity (Fig. 17B) and spared white matter volume (Fig. 17C) were calculated. CHPG infusion resulted in a significant reduction in cavitation, supporting MRI findings. CHPG treatment also increased remaining white matter around the lesion area.

Representative images obtained from the lesion epicentre. Bars represent mean $\pm$ SEM; * p <0.05. Scale bar = 500 μ m.

Figures 18A-D show mGluR5 activation reduces Iba-1 staining after spinal cord injury in a dose-dependent manner. At 7 days post-injury, spinal cord tissue at the lesion site was immunolabeled with Iba-1, a marker for activated microglia/macrophages. Immunolabeling was greatest in tissue that received infusion of vehicle (Fig. 18A). Infusion of 10mM CHPG reduced Iba-1 immunostaining (Fig. 18B). Immunostaining was further reduced following treatment with 41mM CHPG (Fig. 18C). Quantification of immunolabeling revealed a dose-dependent trend with CHPG treatment, and 41mM CHPG significantly reduced immunopositive pixel density in comparison to vehicle-treated tissue (Fig. 18D). Representative images obtained at 1mm caudal to lesion epicenter. Scale bar = 500 μ m. Bars represent mean $\pm$ SEM; * p <0.05.

Figure 19 is a graph showing that mGluR5 activation reduces TNF-alpha secretion at 24 hours after spinal cord injury. TNF-alpha was measured by ELISA in the spinal cord tissue 24 hours after contusion injury. TNF-alpha was significantly increased in animals receiving contusion injury and vehicle treatment in comparison to sham-injured rats. Infusion of CHPG into the spinal cord at 30 minutes after injury resulted in a significant reduction in TNF-alpha protein detection. Bars represent mean $\pm$ SEM; * p <0.05.

Figures 20A-D are graphs showing mGluR5 activation alters the inflammatory response after spinal cord injury (SCI). Microglial-related inflammatory products p22^{phox} (Fig. 20A), ED1 (Fig. 20B), Galectin-3 (Fig. 20C) and iNOS (Fig. 20D) were found to be significantly suppressed 7 or 28 days after SCI in animals treated with CHPG in comparison to control, as measured by Western blotting. Representative Western blots for 7 or 28-day samples are shown. Bars represent mean $\pm$ SEM; * p <0.05.

Figure 21 is a graph showing quantification of ED1 positive pixel density at 28 days after spinal cord injury. mGluR5 activation reduced ED1 expression at 28 days after spinal cord injury. Vehicle treated tissue demonstrated a large amount of staining for ED1, which was decreased in CHPG-treated tissue.

Figure 22 is a graph showing the quantification of gp91^{phox} positive pixel density that revealed a significant reduction in CHPG-treated tissue at 28 days post-injury.

Figures 23A and B are graphs showing that CHPG inhibits LPS-induced activation in spinal cord derived microglia. Spinal cord microglial activation was measured by proliferation (MTS assay, (Fig. 23A) and NO production (Fig. 23B) at 24 hours after stimulation. Both measurements were significantly inhibited by pre-treatment with the mGluR5 agonist, CHPG (100 μ M). Bars represent mean $\pm$ SEM; **p<0.01 vs. control; #p<0.05 vs. LPS.

Figure 24 is a graph showing that CHPG inhibits microglial-induced neurotoxicity. Neuronal number was measured by counting NeuN+ cells 24 hours after microglia/neuron co-culture. The number of NeuN+ cells was reduced by the co-incubation of LPS-stimulated microglia with neurons; this was reversed by the pre-treatment of microglia with CHPG. Addition of the mGluR5 antagonist, MTEP (100 μ M), inhibited the effect of CHPG on microglial-induced neurotoxicity, demonstrating an mGluR5-mediated effect by CHPG. Bars represent mean $\pm$ SEM; *p<0.05 vs. control; #p<0.05 vs. LPS; +p<0.05 vs. CHPG + LPS.

Figures 25A and B are graphs showing results of beam walk and water maze tests for mice with traumatic head injury treated with CHPG thirty minutes following the injury.

Figure 26 is a graph showing lesion volume as determined by MRI 21 days following traumatic brain injury.

Figure 27 is a graph showing results of beam walk tests for mice with traumatic brain injury treated with CHPG 28 days following the injury.

Figure 28 is a graph showing lesion volume as determined by MRI at 1 month, 2 months, and 3 months following traumatic brain injury.

Figure 29 are representative T2 weighted MRI images taken 3 months following traumatic brain injury in animals treated with CHPG at 28 days post-injury.

DETAILED DESCRIPTION

Although the presence of mGluR5 mRNA has been observed in microglia, neither protein expression nor its role in microglial activation and neuroinflammation has been evaluated. Previous studies have reported the presence of mGluRs on

immune cells such as microglia, macrophages and lymphocytes. mGluR5 mRNA has been demonstrated in microglia. This disclosure shows that in a purified rat microglia culture system, microglia express mGluR5 constitutively (Fig. 1), similar to T lymphocytes, while the other group I mGluR, mGluR1, is barely expressed.

5 Although mGluR1 and mGluR5 receptors share certain common signal transduction mechanisms, they have remarkably different profiles in CNS injury. Agonists of mGluR1 exacerbate necrotic cell death, whereas selective antagonists are neuroprotective *in vitro* and *in vivo*. In contrast, activation of mGluR5 inhibits caspase dependent neuronal apoptosis in cell culture models. Although mGluR5
10 antagonists have been reported to be neuroprotective, mGluR5 knockout technology showed such effects are unrelated to actions at the mGluR5 receptor and instead are mediated by direct actions on the NMDA receptor.

 mGluR5 stimulation, however, as shown herein, inhibits microglial activation *in vitro*. Markers of microglial activation, including proliferation, NO, TNF α and
15 ROS production and Galectin-3 expression, as well as microglial-induced neurotoxicity were significantly attenuated by pre-treatment with the mGluR5 agonist CHPG. To verify that these effects were mediated by the mGluR5 receptor, rather than a non-receptor related mechanism, CHPG's effects were shown to be lost in microglia from mGluR5 knockout mice and markedly attenuated by a selective
20 mGluR5 receptor antagonist.

 NO, TNF α and ROS each have been shown to contribute to neuronal damage and death *in vitro* and *in vivo*. For example, ROS induces lipid peroxidation, which results in cell membrane damage and cell death, as well as glutamate release to contribute to further excitotoxic cell death. NO is also associated with neuronal death
25 *in vitro* and *in vivo*, through the production of the toxic metabolite peroxynitrite or via direct action on lipid membranes of the cell or mitochondria. TNF α can cause cell death directly by binding to neuronal TNF receptors linked to death domains that activate caspase-dependent apoptosis. This cytokine can also induce glutamate release and enhance excitotoxicity. In addition, TNF α can induce additional release
30 of ROS, by inducing NADPH oxidase activity. LPS-stimulated microglia induced neuronal cell death, which was attenuated by CHPG treatment. Early suppression of TNF α is protective, whereas knockout of the receptor exacerbates damage after central nervous system injury. In this regard, it should be noted that CHPG treatment

of microglial cells markedly suppressed early TNF α release yet had modest to no effect on later cytokine levels. Finally, Galectin-3, a marker of microglial activation, was also up-regulated after LPS stimulation but reduced by CHPG treatment. Galectin-3 is a carbohydrate binding protein that is up-regulated following injury and plays a role in phagocytosis and perpetuation of the inflammatory response.

In lymphocytes, mGluR5 is coupled to a G_{as}-protein, with activation resulting in cAMP production. Treatment of microglia with CHPG induces cAMP production, and that other cAMP inducers, forskolin and dbcAMP, have similar effects on microglial activity. However, blocking the production of cAMP does not mitigate the effects of CHPG, showing that cAMP is not a critical component in the CHPG signal transduction pathway in microglia.

In neurons and astrocytes, mGluR5-mediated signal transduction involves activation of PLC and PKC, increased inositol triphosphate (IP3), and the release of intracellular calcium. Inhibitors of PLC (U-73122) and PKC (R0318220), as well as calcium chelation (BAPTA-AM), blocked CHPG's effects, showing that CHPG is operating through the PLC/PKC/calcium pathway. In the PLC/PKC pathway, activation of PLC leads to DAG synthesis and IP3 mediated release of Ca²⁺. Both DAG and Ca²⁺ are capable of activating PKC, which has a multitude of actions within the cell, including activation of transcription factors and alteration of potassium channel activity, which may alter microglial responses. Notably, although R0318220 is a PKC inhibitor, it also inhibits the activity of mitogen-activated protein kinase phosphatase-1 (MKP-1). The latter enzyme is induced by TNF α and operates to inactivate mitogen-activated protein kinases and JNK. Without meaning to be limited by theory, as these actions can serve to modulate inflammation, they may reflect another potential mechanism of CHPG action within microglia.

PKC is also involved in the regulation of COX2 in activated microglia and inhibitors of PKC $_{\alpha/\beta II}$ reduce microglial activation. As shown herein, the pan-PKC inhibitor R0318220 at the concentrations used (1.5 μ M) did not have an effect on microglial production of NO or on microglial proliferation but did reverse the effect of CHPG. Thus, different PKC isoforms may be involved in these two systems, and work has suggested that mGluR5 involves phosphorylation of the PKC $_{\epsilon}$ isoform.

Although CHPG provides protection in a rat model of focal brain ischemia, no microglial or mGluR5 receptor mediated reduction in the post-injury inflammatory

response was noted. As shown herein, mGluR5 agonists like CHPG limit microglial activation after central nervous system injury such as traumatic brain or spinal cord injury. Moreover, as mGluR5 stimulation also exerts anti-apoptotic effects on neurons, such treatment has multi-potential neuroprotective actions. As both
5 neuroinflammation and caspase-dependent neuronal apoptosis have been implicated in many acute and chronic neurodegenerative disorders, mGluR5 agonist therapy has broad therapeutic relevance.

Thus, provided herein is a method of reducing or preventing neuroinflammation or neurotoxicity in a subject. The method includes administering
10 to the subject an mGluR5 agonist. Optionally the agonist is CHPG, but in certain aspects may be agonists other than CHPG. The mGluR5 agonist can be a small molecule or a polypeptide (e.g., a fragment of the mGluR5 that competes for ligand binding or an agonistic antibody (or fragment thereof) to mGluR5). Optionally, the agonist is formulated to ensure that it crosses the blood brain barrier (BBB). For
15 example, the agonist can be formulated in a liposome. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs (targeting moieties), thus providing targeted drug delivery. Exemplary targeting moieties include folate, biotin, mannosides, antibodies, surfactant protein A receptor and gp120. To ensure that agents cross the BBB, they may also be coupled to a BBB
20 transport vector (see Bickel, et al., Adv. Drug Delivery Reviews, vol. 46, pp. 247-279, 2001). Exemplary transport vectors include cationized albumin or the OX26 monoclonal antibody to the transferrin receptor; these proteins undergo absorptive-mediated and receptor-mediated transcytosis through the BBB, respectively.

The mGluR5 agonist is administered by any route determined by the skilled
25 artisan to be effective. For example, the agonist is systemically administered to the subject. Alternatively, the agonist is administered intraventricularly to the subject. The agonists can be administered directly into the central nervous system of the subject. The later option is likely to be associated with surgical intervention and thus may be used in combination with neurosurgery to reduce inflammation and
30 neurotoxicity associated with such surgery.

According to the methods taught herein, the subject is administered an effective amount of the agonist. The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary

to produce a desired physiologic response. Effective amounts and schedules for administering the agonist may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration are those large enough to produce the desired effect in which the symptoms or disorder are affected. The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products.

The subject to be treated optionally has an central nervous system injury or central nervous system disease or disorder such as a neurodegenerative disease. Central nervous system injury include spinal cord injury, stroke, head injury. The injury can be acute or chronic.

The subject to be treated may be at risk for or suspected of having a neurodegenerative disorder, including, for example, Alzheimer's Disease, Parkinson's Disease, and multiple sclerosis. A subject suspected of having or being at risk for a central nervous system disease or disorder can be determined by one of skill in the art risk based on, for example, a history of head trauma, a family history, or early signs and symptoms (e.g., tremor, memory deficits, etc.).

Optionally, the administration of the mGluR5 agonist is used in combination with other therapeutic or prophylactic regimens. Illustrative examples of therapeutic agents include, but are not limited to, anti-inflammatory agents, antibiotics, immunosuppressive agents, and immunoglobulins. Optionally, the provided agonist is administered in combination with other neuroprotective compounds. The agonist can be administered in combination with a chemotherapeutic agent and radiation or with neurosurgery. Other combinations of treatment modalities can be administered as desired by those of skill in the art. Combinations may be administered either concomitantly (e.g., as an admixture), separately but simultaneously (e.g., via separate intravenous lines into the same subject), or sequentially (e.g., one of the compounds

or agents is given first followed by the second). Thus, the term combination is used to refer to either concomitant, simultaneous, or sequential administration of two or more agents.

Also provided is a method of reducing activation of a microglial cell comprising contacting the microglia cell with an mGluR5 agonist. The contacting step can be performed *in vitro* or *in vivo*.

Also provided herein are methods of screening for agents that reduce neuroinflammation or neurotoxicity comprising contacting a microglial cell with an agent to be tested and an inflammatory stimulus and detecting the level of NO, ROS, TNF α , or microglial proliferation, with a reduced level of NO, ROS, TNF α , or microglial proliferation compared to the inflammatory stimulus alone indicating the agent reduces or prevents neuroinflammation or neurotoxicity. An inflammatory stimulus, for example, can be LPS treatment.

As used herein, the term peptide, polypeptide, protein or peptide portion is used broadly herein to mean two or more amino acids linked by a peptide bond. Protein, peptide and polypeptide are also used herein interchangeably to refer to amino acid sequences. The term fragment is used herein to refer to a portion of a full-length polypeptide or protein. It should be recognized that the term polypeptide is not used herein to suggest a particular size or number of amino acids comprising the molecule and that a peptide of the invention can contain up to several amino acid residues or more.

The term antibody is used herein in a broad sense and includes both polyclonal and monoclonal antibodies. The term can also refer to a human antibody and/or a humanized antibody. Examples of techniques for human monoclonal antibody production include those described by Cole et al. (Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985) and by Boerner et al. (J. Immunol., 147(1):86-95, 1991). Human antibodies (and fragments thereof) can also be produced using phage display libraries (Hoogenboom et al., J. Mol. Biol., 227:381, 1991; Marks et al., J. Mol. Biol., 222:581, 1991). The disclosed human antibodies can also be obtained from transgenic animals. For example, transgenic, mutant mice that are capable of producing a full repertoire of human antibodies, in response to immunization, have been described (see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-2555

(1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immunol., 7:33 (1993)).

As used herein the terms treatment, treat or treating refer to a method of reducing the effects of a disease or condition or symptom of the disease or condition. Thus in the disclosed method treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% reduction in the severity of an established disease or condition or symptom of the disease or condition. For example, the method for treating a protein aggregate disorder is considered to be a treatment if there is at least a 10% reduction in one or more symptoms of the disease in a subject as compared to control. Thus the reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% or any percent reduction in between 10 and 100 as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disease, condition or symptoms of the disease or condition.

As used herein, the terms prevent, preventing and prevention of a disease or disorder refers to an action, for example, of administration of a therapeutic agent, that occurs before a subject begins to suffer from one or more symptoms of the disease or disorder, which inhibits or delays onset of one or more symptoms of the disease or disorder.

As used herein, subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, pig, rabbit, dog, sheep, goat, non-human primate, cow, cat, guinea pig or rodent), a fish, a bird or a reptile or an amphibian. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject afflicted with a disease or disorder (e.g., Parkinson's Disease). The term patient or subject includes human and veterinary subjects.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For

example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary.

5 Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods of using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific method steps or combination of
10 method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

EXAMPLES

15 Example 1

Materials and Methods

Microglial cultures and treatment

Microglial cells were obtained from postnatal day 2 Sprague Dawley rat pups, day 2 mGluR5 knockout and wild-type mouse pups, or day 2 C57Bl6 mouse pups and
20 cultured as described in Byrnes et al., Glia 53(4):420-33 (1996). The whole brain was carefully dissected and homogenized in L15 media (Gibco; Carlsbad, CA). Mixed glial cultures were incubated for 8–10 days at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Media (Gibco) with 10% Fetal Calf Serum (Hyclone; Logan, UT), 1% L-glutamine (Gibco), 1% Sodium Pyruvate (Gibco), and 1% Pen/Strep (Fisher;
25 Pittsburgh, PA). After the initial incubation, the cells were shaken for 1 hour at 100 rpm and 37°C. Detached microglia were collected and replated as purified cultures with greater than 96% purity.

CHPG ((RS)-2-chloro-5-hydroxyphenylglycine; Tocris; Ellisville, MO) was applied to cells for 1 hour prior to lipopolysaccharide (LPS; 100ng/ml) stimulation.
30 The mGluR5 antagonist MTEP (3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; 100μM) was a gift from Merck Research Laboratories (Rahway, NJ), and was administered 30 minutes prior to CHPG administration. Forskolin (50μM, Tocris)

and dibutyl cAMP (200 μ M, BioMol; Plymouth Meeting, PA) were administered 1 hour prior to LPS stimulation. SQ22356, RpcAMPs (Calbiochem; San Diego, CA), R0318220, U-73122 (10 μ M; BioMol; Plymouth Meeting, PA), and BAPTA-AM (1.5 μ M; Molecular Probes; Carlsbad, CA) were administered 30 minutes prior to CHPG administration. All chemicals were prepared and stored according to the manufacturer's guidelines.

Immunolabeling of microglia

At 24 hours post-purification, microglia in 24-well plates on glass cover-slips were stimulated with LPS (100 ng/ml) or were untreated (control) for an additional 24 hours. Cells were then fixed in 4% paraformaldehyde and subjected to standard immunohistochemistry, performed as described Byrnes et al., *Glia* 53(4):420-33 (1996) using antibodies against mGluR1 α and mGluR5 (Chemicon; Billerica, MA), OX42 (Serotec; Raleigh, NC), ED1 (Serotec), and Galectin-3 (Abcam; Cambridge, MA). Confocal fluorescence microscopy imaging was performed using Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss; Thornwood, NY).

Microglial proliferation and viability

At various time points after application of mGluR agonists/antagonists, proliferation of microglia in 96-well plates was assessed using the MTS assay (MTS tetrazolium compound; Cell Titer 96® Aqueous One Solution, Promega; Madison, WI); cell death was assessed with the lactate dehydrogenase (LDH) release assay (CytoTox 96® nonradioactive cytotoxicity assay kit, Promega; Madison, WI).

Nitric Oxide production

Nitric oxide (NO) production was assayed using the Griess Reagent Assay (Invitrogen; Carlsbad, CA).

TNF α detection

TNF α protein secreted into the media was assessed using the TNF α ELISA kit (Endogen; Rockford, IL).

ROS detection

Intracellular reactive oxygen species (ROS) production was assessed at 24 hours after stimulation by measuring the oxidation of 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H2DCFDA; Molecular Probes; Eugene, OR). Media from microglia plated into 96-well plates was aspirated and

replaced with warmed 1X PBS. CM-H2DCFDA (10 μ M) was added to microglia and incubated for 45 minutes. Fluorescence was measured using excitation and emission wavelengths of 490 and 535 nm, respectively.

Neurotoxicity

5 Rat primary cortical neuronal cultures were derived from E18 rat cortices (Taconic; Germantown, NY) as previously described in Mukin et al., J Neurosci Res 51(6):748-58 (1998). At 24 hours after stimulation, microglia in transwell inserts were washed in media and inserted into 24-well plates containing neurons (neurons were at day 5 *in vitro*). Twenty-four hours later, microglia were removed and the
10 LDH release assay was used to assess neuronal cell death.

³H-PI Hydrolysis assay

Primary microglia were cultured in 96-well plates and incubated overnight with 0.625 μ Ci/well myo-[³H]inositol (NEN, Boston, MA) to label the cell membrane phosphoinositides and perform the assay, as described previously in Surin et al.,
15 Neuropharmacology 52(3): 744-54 (2007). Briefly, cells were washed in Locke's buffer and incubated with or without 100 μ M CHPG for 1 hour at 37 °C in Locke's buffer containing 20 mM LiCl to block inositol phosphate degradation. Inositol phosphates were then extracted with 0.1 M HCl for 10 minutes. The separation of [³H]inositol phosphates was performed by ion-exchange chromatography on AG 1-X8
20 resin (200–400 mesh; Bio-Rad Laboratories; Hercules, CA). Total [³H]inositol phosphates were eluted from the columns with 0.5 ml of 0.1 M formic acid/1 M ammonium formate. The collected samples were mixed with Safety-Solve cocktail (RPI; Mount Prospect, IL) and measured by scintillation counting.

Statistical analysis

25 Quantitative data are presented as mean +/- SEM. Data were analyzed using Student's t-test or one-way ANOVA, where appropriate. All statistical tests were performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc.; San Diego, CA). A p value < 0.05 was considered statistically significant.

Results

Microglia express mGluR5

Western blot analysis demonstrated that mGluR5 protein is highly expressed in rat whole brain microglial cultures whereas expression of the other group I mGluR, mGluR1, is barely detectable in these cells (Fig. 1). Rat cortical neuron (RCN) samples, which constitutively express all mGluR receptors, were run alongside as positive controls for the antibodies.

Immunocytochemistry was also used to confirm mGluR5 expression in microglial cultures. Immunolabeling for mGluR1 α revealed no receptor expression on microglia. Strong mGluR5 immunolabeling was detected on the surface of primary microglia. mGluR5 labeling is found particularly on the cell periphery, consistent with membrane localization. Further, to confirm microglial expression, double-labeling was performed with common markers of microglia and mGluR5. mGluR5 was expressed on cells that were also positive for OX42 and ED1.

mGluR5 stimulation reduces microglial activation

In order to determine if microglia respond to mGluR5 stimulation, microglia were cultured in 96-well plates and mGluR5 agonists/antagonists were added alone or in combination to determine their effect on microglial activation. Pre-treatment with CHPG was found to reduce expression of several independent markers of microglial activation, including ROS production, NO production, proliferation and TNF α production.

CHPG's effects were determined to be dose dependent, with maximal effects at 100 μ M, as measured by both ROS and NO production ($p < 0.05$; Fig. 2A, B). CHPG addition without LPS stimulation had no effect on microglial ROS or NO production (Fig. 2A, B).

Addition of LPS (100ng/ml) to microglia resulted in an increase in proliferation over 24 hours, which was blocked by 1 hour pre-treatment with CHPG (100 μ M; $p < 0.05$) (Fig. 3A). NO production was also significantly increased by 15 and 24 hours after LPS stimulation (Fig. 3B). Pre-treatment with CHPG significantly attenuated the LPS induced increase in NO production by 24 hours ($p < 0.05$; Fig. 3B). TNF α production was up-regulated by LPS stimulation within 30 minutes and remained at increased levels through 24 hours ($p < 0.001$; Fig. 3C). CHPG pre-

treatment significantly attenuated TNF α production from 30 minutes through 8 hours, delaying the increase in TNF α observed after LPS stimulation.

Galectin-3, a marker of microglial activation was also expressed when microglia were stimulated with LPS. Pre-treatment with CHPG resulted in a loss of Galectin-3 staining, similar to control cultures.

mGluR5 stimulation reduces neurotoxicity

Addition of activated microglia to neuronal cultures is known to induce neuronal cell death. In order to determine if mGluR5 plays a role in microglial-induced neurotoxicity, agonists/antagonists were added to microglia prior to co-culture with neurons. Stimulation of microglia with LPS prior to co-culture significantly decreased neuron viability, as measured by neuronal LDH release ($p < 0.05$; Fig. 4).

Pre-treatment of microglia with CHPG prior to LPS addition resulted in a significant increase in neuronal viability ($p < 0.05$; Fig. 4). In order to eliminate the possibility of a direct effect of CHPG on neurons in this study, microglia were washed prior to addition to neurons. Neither LPS nor CHPG had any direct effect on neurons when added without microglia. Addition of the selective mGluR5 antagonist MTEP (100 μ M) prior to CHPG pre-treatment reversed the protective effect of CHPG, reducing neuronal viability ($p < 0.05$; Fig. 4), suggesting that CHPG acts through the mGluR5 receptor.

The mGluR5 receptor is necessary for the action of CHPG

To confirm that CHPG is acting through the mGluR5 receptor, microglia from mGluR5 knockout (-/-) and wild-type (+/+) mice were obtained and stimulated with LPS. LPS stimulation increased microglial proliferation, NO production and ROS production in both knockout and wild-type cells (Fig. 5). However, one hour pre-treatment of microglia with CHPG attenuated the effects of LPS in wild-type microglia (Figs. 5A, C, E) but not those from mGluR5 knockouts (Figs. 5B, D, F), thus demonstrating that CHPG acts through mGluR5 receptor activation.

mGluR5 activation results in cAMP production

In T-lymphocytes, activation of mGluR5 receptors induces activation of adenylyl cyclase, cAMP production and PKA induction. To determine if this pathway is utilized in microglia, cAMP was measured in microglia following CHPG

application. CHPG significantly increased cAMP production by 24 hours (Fig. 6A). To further investigate the potential role of cAMP in microglial activation, cAMP inducers, forskolin (50 μ M) and dibutyryl cAMP (dbcAMP; 200 μ M), were applied to microglia prior to LPS stimulation; each caused suppression of microglial activation as measured by NO production, similar to that of CHPG ($p < 0.05$; Fig. 6B).

In order to determine if this pathway was required for CHPG's activities in microglia, cells were pre-treated with the adenylyl cyclase inhibitor SQ22356 (0.1 – 500 μ M) or the PKA inhibitor Rp-cAMPs (10 – 500 μ M) prior to addition of CHPG. Pre-treatment with either inhibitor failed to induce any significant reduction in CHPG's effects on microglial activation, as measured by ROS production (Fig. 6C, D), suggesting that cAMP production is not necessary for CHPG's activity in microglial cells.

Inhibition of phospholipase C blocks the effects of CHPG

In neurons and astrocytes, mGluR5 stimulation results in $G_{\alpha q}$ activation, triggering PLC phosphorylation, hydrolysis of phosphatidyl inositol (PI), PKC activation and calcium release. In order to determine if these signal transduction pathways were involved in CHPG signalling in microglia, hydrolysis of PI was assessed. CHPG treatment resulted in a significant increase PI hydrolysis ($p < 0.01$; Fig. 7A), suggesting the activation of $G_{\alpha q}$. To further assess the role of this pathway, cells were pre-treated with inhibitors of PLC (U-73122) and PKC (R0318220) prior to CHPG and LPS addition. Using microglial activation outcomes such as ROS production, NO production and proliferation, U-73122 and R0318220 were found to block the effects of CHPG, returning NO, ROS and proliferation to LPS-induced levels (Fig. 7B, C, D). The calcium chelator (BAPTA-AM) also reversed the effects of CHPG (Fig. 7C). These inhibitors did not affect the activity of LPS or of microglia alone when introduced without CHPG pre-treatment, demonstrating that their activities were specific for the CHPG signal transduction pathway.

Example 2

Materials and Methods

Microglial cultures

Primary cortical microglial cells were obtained from postnatal day 2 Sprague Dawley rat pups and cultured. 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 with 5% CO₂ in Dulbecco's Modified Eagle Media (DMEM; Gibco Invitrogen) 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). After the initial incubation, the cells were shaken for 1 hr at 100 rpm and 37°C. Detached microglia were collected and replated as purified cultures with greater than 96% purity. The cells were grown and maintained in DMEM supplemented with 10% Fetal Bovine Serum (Hyclone) at 37°C in a humidified incubator under 5% CO₂.

Drug treatments

The mGluR5 agonist, CHPG ((RS)-2-chloro-5-hydroxyphenylglycine; 100µM-10mM), group 1 mGluR agonist, DHPG ((RS)-3,5-Dihydroxyphenylglycine; 50µM), and the mGluR1 antagonist, CPCCOEt, (7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; 100µM; all from Tocris Bioscience, Ellisville, MO) were applied alone and/or in combination to microglia for 1 hr prior to lipopolysaccharide (LPS (Sigma-Aldrich); 100ng/ml) or recombinant mouse interferon-γ (IFNγ (R&D Systems, Minneapolis, MN); 0.5 and 2ng/ml) stimulation. The mGluR5 antagonist, MTEP (3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; 10µM) (Merck Research Laboratories (Rahway, NJ)) was administered 30 min prior to CHPG administration. All drugs were prepared and stored according to the manufacturer's guidelines.

RNA interference

Small interfering RNA (siRNA) containing a mixture of three targeted siRNAs for mouse mGluR5, p22^{PHOX}, and gp91^{PHOX} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BV2 microglia cultured in 24-well plates were transfected with the appropriate siRNA (100nM) using Lipofectamine2000® (Invitrogen, Carlsbad, CA). After 24 hrs of transfection, cells were pre-treated with CHPG (4mM) for 1 hr, stimulated with LPS (100ng/ml) and cultured for an additional 24 hrs. Control siRNA duplex containing scrambled sequences (Santa Cruz Biotechnology, Santa Cruz, CA) was used in parallel experiments. Optimal transfection efficiency and conditions were determined by using fluorescein-labelled dsRNA oligomers (BLOCK-iT® Fluorescent Oligo; Invitrogen, Carlsbad, CA). The

transfection efficiency of siRNA in BV2 microglia was approximately 50% as determined by fluorescently-labeled cells containing the fluorescent-oligo siRNA 24 hrs after transfection. Effective gene knockdown was analyzed by Western immunoblotting.

5 Immunocytochemistry

BV2 and primary cortical microglia were seeded onto poly-D-lysine coated coverslips in 24-well plates at a density of 8×10^5 cells/well. After 24 hrs, cells were pre-treated for 1 hr with CHPG, stimulated with LPS and incubated for an additional 24 hrs. Cells were washed in warm PBS and fixed in 4% paraformaldehyde for 15
10 mins followed by three washes with PBS for 10 mins each. Cells were incubated in blocking buffer (10% normal goat serum in PBS containing 0.1% Triton X100) for 2 hrs, followed by overnight incubation at 4°C in primary antibody (mGluR5; 1:100, (Abcam, Cambridge, MA), ED1; 1:100, (AbD Serotec, Raleigh, NC), p22^{PHOX}; 1:50, (Santa Cruz Biotechnology, Santa Cruz, CA) and gp91^{PHOX}; 1:100, (BD Transduction
15 Laboratories, Franklin Lakes, NJ)). The next day, cells were washed with PBS three times for 10 mins, followed by appropriate secondary antibodies (Alexa Flour 488 and Alexa Flour 546; 1:1000, (Molecular Probes, Carlsbad, CA)) for 1 hr at room temperature, and three washes with PBS. The coverslips were inverted and mounted on a slide using Hydromount mounting media. Confocal fluorescence microscopy
20 imaging was performed using Zeiss 510 Meta® confocal laser scanning microscope (LSM 510 META® (Zeiss, Oberkochen, Germany). Visualization of the fluorophores was achieved using the 488-nm argon laser, and a 543-nm helium/neon laser.

Measurement of PI hydrolysis

BV2 microglia, cultured in 96-well plates, were incubated overnight with
25 0.625 µCi/well myo-[³H]inositol (NEN, Boston, MA) to label the cell membrane phosphoinositides. After two washes with Locke's buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1 mM MgCl₂, 1.3 mM CaCl₂, 5.6 mM glucose and 20 mM Hepes, pH 7.4) incubations with CHPG were carried out for 1 hr at 37°C in Locke's buffer containing 20 mM LiCl to block inositol phosphate degradation. The reaction
30 was terminated by aspiration of media and inositol phosphates were extracted with 0.1 M HCl for 10 min. The separation of [³H]inositol phosphates was performed by ion-exchange chromatography on AG 1-X8 resin (200–400 mesh; Bio-Rad Laboratories,

Hercules, CA). The samples were diluted 10 times with water and applied to columns equilibrated in 0.1 M formic acid. The columns were washed with 1 ml of water and 1 ml of tetraborate buffer (5 mM sodium tetraborate, 60 mM sodium formate). Total [³H]inositol phosphates were eluted from the columns with 0.5 ml of 0.1 M formic acid/1 M ammonium formate. The collected samples were mixed with Safety-Solve® cocktail (RPI, Mount Prospect, IL) and measured by scintillation counting.

Western immunoblot analysis

BV2 and primary cortical microglia, cultured in 6-well dishes, were pre-treated for 1 hr with CHPG, stimulated with LPS and incubated at 37°C and 5% CO₂ for the indicated time. Cells were harvested by scraping with a cell scraper and maintained on ice. Samples were washed once with ice-cold PBS and centrifuged at 2,000 × g for 3 min. The cellular pellet was resuspended in lysis buffer (60 mM Tris-HCl, pH 7.8 containing 150mM NaCl, 5mM EDTA, 10% glycerol, 2mM Na₃VO₄, 25mM NaF, 10µg/ml leupeptin, 10µg/ml aprotinin, 1mM AEBSF, 1mM pepstatin, 1µM microcystin LR (all Sigma-Aldrich, St Louis, MO), and 1% Triton X-100 (Calbiochem, La Jolla, CA)). The samples were lysed on ice for 30 min and centrifuged at 20,000 × g for 15 min. The soluble fraction containing total cell extracts was recovered, the protein concentration determined and samples were equalized. Protein samples were resolved by 8-15% SDS-PAGE (Mini-Protein 3®, Bio-Rad Laboratories, Hercules, CA), transferred onto nitrocellulose membrane (Optitran® BA-S 85, Whatmann, Dassel, Germany) and blocked for a minimum of 1 hr in blocking buffer (5% Skimmed Milk in PBS containing 0.05% Tween-20 (PBS-T)). Membranes were incubated overnight at 4°C with antibodies for mGluR5 (1:1000, Abcam, Cambridge, MA), mGluR1α (1:1000; Chemicon International, Billerica, MA), iNOS (1:1000; BD Transduction Laboratories, Franklin Lakes, NJ), p22^{PHOX} (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), gp91^{PHOX} (1:1000; BD Transduction Laboratories) and β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO) in PBS-T containing 1% skimmed milk. Membranes were washed (4 × 10 min in PBS-T), incubated in the appropriate horseradish peroxidase conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG, 1:2000; Jackson ImmunoResearch laboratories, West Grove, PA) for 1 hr at room temperature. Membranes were washed and protein complexes were visualized using SuperSignal West Dura

Extended Duration Substrate (Pierce, Rockford, IL). Protein immunoblots were exposed to X-ray film (RPI Corp., Mt. Prospect, IL) and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis using QuantityOne Basic software (Bio-Rad Laboratories, Hercules, CA). The data presented represent the density of target protein divided by the density of the endogenous β -actin in each sample and are expressed in arbitrary units.

Nitric Oxide assay

Nitric oxide (NO) production was assayed using the Griess® Reagent Assay (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

TNF α assay

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for detecting TNF α (R&D Systems, Minneapolis, MN) in culture supernatants. Assays were performed as per manufacturer's instructions. Cytokine concentrations were calculated using standard curves generated from recombinant mouse TNF α , and the results were expressed in pg/ml.

Measurement of intracellular ROS

Intracellular ROS levels were measured by 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA). BV2 microglia were pre-treated with CHPG (4mM) or apocynin (1mM) and stimulated with LPS (100ng/ml) for 24 hrs. The cells were incubated with 10 μ M H₂DCFDA (Molecular Probes, Eugene, OR) for 45 min at 37°C in 5% CO₂. Fluorescence was measured using excitation and emission wavelengths of 490 and 535 nm, respectively. Data are presented as percentage of control-treated values.

NADPH Oxidase activity assay

NADPH oxidase activity was assessed using a DHE (Dihydroethidium)-derived fluorescence assay for NADPH oxidase in the microplate reader. Briefly, BV2 microglia, cultured in 6-well plates, were pre-treated with CHPG (4mM) or apocynin (1mM) and stimulated with LPS (100ng/ml) for 4 and 24 hrs. Cell membrane homogenates were obtained by centrifugation at 18,000 x g for 15 min to separate mitochondria and nuclei, and the supernatant was further centrifuged at 100,000 x g for 1 hr to obtain a membrane-enriched fraction. Membrane fractions (10 μ g) were incubated with DHE (10 μ M) and DNA (1.25 μ g/ml) in PBS/DTPA with

the addition of NADPH (50 μ M), at a final volume of 120 μ l for 30 min at 37°C in the dark. Fluorescence emissions were followed in a cytofluorometer (excitation 490 nm and emission 590 nm). Data are presented as percentage of control-treated values.

Neurotoxicity assay

5 BV2 microglia were pre-treated for 1 hr with CHPG (4mM), stimulated with LPS (100ng/ml) and incubated at 37°C and 5% CO₂ for 24 hrs. Conditioned media from BV2 microglia was added to confluent B35 neuroblastoma cells and the cells were incubated at 37°C and 5% CO₂ for a further 24 hrs. Cell-death was measured by lactate dehydrogenase (LDH) release assay (CytoTox96™ non-radioactive
10 cytotoxicity assay, Promega, Madison, WI) according to the manufacturer's instructions. Neuronal cell-death was determined by subtracting the LDH values in conditioned BV2 media (day 1) from LDH values in B35 neuroblastoma media (day 2).

Statistical analysis

15 For statistical analysis, data obtained from independent measurements were presented as the mean $\pm$ SEM and they were analyzed using a Student's *t* test or ANOVA followed by the post-hoc Newman-Keuls Multiple Comparison Test. All statistical tests were performed using the GraphPad® Prism Program, Version 3.02 for Windows® (GraphPad Software, Inc. San Diego, CA). Differences were
20 considered significant for $p < 0.05$.

Results

Microglia express functional mGluR5 receptors

To demonstrate the expression of mGluR5 on microglia, immunohistochemical studies using antibodies directed against mGluR5 and ED-1, a
25 marker of activated microglia, were performed on BV2 microglia that had been cultured in the presence or absence of LPS (100ng/ml) for 24 hrs. Strong mGluR5 immunolabelling was observed in control- and LPS-stimulated microglia, and LPS-treatment transformed the microglia from a predominantly resting, flat cell morphology to an activated, amoeboid-shaped morphology with intensified ED-1
30 immunoreactivity. Western immunoblot analysis confirmed that mGluR5 was clearly expressed in BV2 microglia under control- and LPS-stimulated conditions whereas

expression of the other group I mGluR, mGluR1 α , was barely detectable in these cells (Fig. 8A). Similarly, when the expression of these receptors was assessed in cultured primary cortical microglia, mGluR5 was found to be highly expressed in control and LPS-simulated cortical microglia whereas mGluR1 expression was weak and negligible by comparison (Fig. 8A). Classical activation of mGluR5 receptors results in G $_{\alpha q}$ activation, PLC phosphorylation, phosphoinositide (PI) hydrolysis, PKC activation and calcium release. In order to demonstrate that mGluR5 receptors on microglia are functional, signaling mechanisms downstream of the receptor were assessed. Increasing concentrations of (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), a selective mGluR5 agonist, were added to BV2 microglia and hydrolysis of myo-[3 H]inositol was measured after 1hr. CHPG treatment dose-dependently increased PI hydrolysis with a significant increase at 5mM (Fig. 8B, *p<0.05 versus control; ANOVA), demonstrating G $_{\alpha q}$ activation and the presence of functional mGluR5 receptors on microglia.

Selective activation of mGluR5 reduces microglial activation

In order to determine if mGluR5 stimulation can modulate microglial activation, BV2 microglia were cultured in 96-well plates and mGluR5 agonists/antagonists were added alone or in combination for 1 hr and the cells were subsequently stimulated by LPS for a further 24 hrs. NO release from microglia was measured as a classical marker of activation. CHPG, significantly attenuated LPS-stimulated NO production in a dose-dependent manner starting at 1mM (Fig. 9A, $^+p<0.05$, $^{+++}p<0.001$ versus LPS; ANOVA). 4mM CHPG reduced LPS-stimulated NO production by ~50% and this concentration was used for all subsequent experiments. Moreover, a combinatorial pharmacological approach that targeted mGluR5 receptor activation by adding the non-selective group I mGluR agonist DHPG (50 μ M) in the presence of a mGluR1 antagonist, CPCCOEt (100 μ M), also resulted in significant attenuation of NO production in response to LPS-stimulation (Fig. 9B, $^{+++}p<0.001$ versus LPS; ANOVA). In contrast, combinational pharmacology that targeted mGluR1 (DHPG (50 μ M) in the presence of a mGluR5 antagonist, MTEP (10 μ M)) did not affect LPS-stimulated NO production in BV2 microglia. In addition, the mGluR5 antagonist 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP, 10 μ M), reversed CHPG's attenuation of LPS-stimulated

NO production when it was added to the cells prior to CHPG pre-treatment (Fig. 9C, $^{***}p < 0.001$ versus LPS+CHPG; ANOVA), indicating that CHPG acts through the mGluR5 receptor. Application of CHPG or MTEP in the absence of LPS did not result in microglial activation or the release of NO (Fig. 9C). Furthermore, siRNA knockdown of the mGluR5 receptor reduced CHPG's protective effects following LPS-stimulation. In BV2 microglia that expressed scrambled control-siRNA, CHPG pre-treatment resulted in a $57.74 \pm 2.98\%$ reduction in LPS-stimulated TNF α pro-inflammatory cytokine release (Fig. 9D). In contrast, in BV2 microglia expressing mGluR5-siRNA, CHPG pre-treatment resulted in only a $33.18 \pm 1.98\%$ reduction. These data indicate that an approximate 57.46% loss of CHPG's effectiveness was achieved when the mGluR5 receptor was knocked down in BV2 microglia (Fig. 9D, $^{***}p < 0.001$; student's t-test). The incomplete effect of mGluR5-siRNA on CHPG's actions was due to partial mGluR5 protein knockdown (approximately 50%) probably resulting from a 50% transfection efficiency of siRNA oligonucleotides in BV2 microglia. These data indicate that the mGluR5 receptor mediates CHPG's modulatory actions in microglia.

CHPG attenuates the release of LPS-stimulated pro-inflammatory mediators and microglial-mediated neurotoxicity

Upon activation, microglia exert neurotoxic effects by releasing pro-inflammatory mediators such as NO and TNF α . The time-course of microglial activation following LPS-stimulation was assessed to see if mGluR5 activation modulates the release of pro-inflammatory mediators. Expression of iNOS protein was significantly increased after 4 hrs of stimulation, peaked at 15 hrs and remained elevated through 24 hrs of stimulation (Fig. 10Ai, $^{***}p < 0.001$ versus control; ANOVA). Pre-treatment of BV2 microglia with CHPG significantly attenuated the expression of iNOS at 15 and 24 hrs post-treatment ($^{++}p < 0.01$, $^{+++}p < 0.001$ versus LPS). iNOS expression was also assessed in primary cortical microglia: LPS-stimulation resulted in increased iNOS expression after 24 hrs that was significantly reduced by CHPG pre-treatment (Fig. 10Aii, $^{***}p < 0.001$ versus control, $^{+++}p < 0.001$ versus LPS; ANOVA). NO production was monitored over time in BV2 microglia and was significantly elevated after 15 and 24 hrs stimulation with LPS (Fig. 10B, $^{***}p < 0.001$ versus control; ANOVA). As anticipated, CHPG pre-treatment resulted in attenuated levels of NO production at both time points ($^{+++}p < 0.001$ versus LPS)

thereby demonstrating that mGluR5 activation attenuates NO release from microglia after LPS-stimulation. We measured the levels of the pro-inflammatory cytokine, TNF α , over time and observed elevated levels of microglial TNF α release as early as 1 hr post-stimulation with highest TNF α levels between 4 and 24 hrs of stimulation (Fig. 10C, ***p<0.001 versus control; ANOVA). CHPG pre-treatment significantly reduced LPS-stimulated TNF α release from microglia as early as 1 hr post-treatment through 24 hrs post-treatment (⁺⁺p<0.01, ⁺⁺⁺p<0.001 versus LPS). Thus, activation of mGluR5 attenuated microglial activation and reduced the release of pro-inflammatory mediators that are known to induce neuronal cell death. Furthermore, when conditioned media from LPS-stimulated microglia was added to cultured B35 neuroblastoma cells, neuronal cell death, as measured by neuronal LDH release, was significantly increased after 24 hrs (***p<0.001 versus control; ANOVA). However, pre-treatment of microglia with CHPG prior to LPS-stimulation and addition of conditioned media to neurons significantly reduced neuronal cell death (⁺⁺⁺p<0.001 versus LPS), demonstrating the neuroprotective effect of stimulating microglial mGluR5 receptors.

mGluR5 activation inhibits microglial NADPH oxidase activity and LPS-generated ROS

ROS generated by NADPH oxidase is an early microglial response to environmental stresses or immunological challenges that often leads to neurotoxicity. As NADPH oxidase mediates LPS-induced neurotoxicity and pro-inflammatory gene expression in activated microglia, it was investigated whether mGluR5 activation modulates microglial NADPH oxidase activity and the subsequent generation of ROS. The expression of the membrane subunits of the NADPH oxidase complex, p22^{PHOX} and gp91^{PHOX}, was assessed following stimulation with LPS for 24 hrs in BV2 or primary cortical microglia. LPS stimulation increased p22^{PHOX} and gp91^{PHOX} immunostaining that co-localized at the membrane of activated microglia in BV2 and primary cortical cultures. Pre-treatment of microglia for 1 hr with CHPG decreased p22^{PHOX} and gp91^{PHOX} immunostaining that was diffusely distributed throughout the cell, similar to control-treated microglia. Quantitative analysis of p22^{PHOX} and gp91^{PHOX} expression by Western immunoblotting confirmed a significant increase in p22^{PHOX} and gp91^{PHOX} expression after LPS-stimulation (Fig. 11, ***p<0.001 versus Control; ANOVA) that was significantly reduced by pre-treatment with CHPG

($^+p<0.05$ versus LPS). These data demonstrate that mGluR5 activation down-regulates the expression of the NADPH oxidase components p22^{PHOX} and gp91^{PHOX} following LPS-stimulation.

The enzymatic activity of NADPH oxidase was assessed in BV2 microglia that were stimulated for 4 or 24 hrs, with LPS and/or prior pre-treatment with CHPG or the NADPH oxidase inhibitor apocynin (1mM). LPS caused a significant increase in NADPH oxidase activity after 4 hrs of stimulation (Fig. 12A, $***p<0.001$ versus Control; ANOVA) while pre-treatment with CHPG attenuated the activity of NADPH oxidase, returning it to control levels ($^{+++}p<0.001$ versus LPS). Application of the NADPH oxidase inhibitor apocynin prior to LPS stimulation resulted in a comparable reduction in NADPH oxidase activity ($^{+++}p<0.001$ versus LPS). Incidentally, NADPH oxidase activity was also increased after 24 hrs of stimulation but did not reach statistical significance; both treatments reduced NADPH oxidase activity at this time-point. Finally, intracellular ROS generation was measured in BV2 microglia via DCFH oxidation. DCFH-DA enters cells passively and is deacetylated by esterase to nonfluorescent DCFH. DCFH reacts with ROS to form dichlorodifluorescein, the fluorescent product. Intracellular ROS was generated in BV2 microglia that had been stimulated with LPS for 24 hrs (Fig. 12B, $***p<0.001$ versus control; ANOVA). Pre-treatment of microglia with CHPG or apocynin caused a significant, comparable reduction in intracellular ROS production in response to LPS-stimulation ($^{++}p<0.01$ and $^+p<0.05$ versus LPS), and application of CHPG or apocynin in the absence of LPS resulted in no ROS generation. Thus, activation of mGluR5 by CHPG reduces p22^{PHOX} and gp91^{PHOX} expression and membrane co-localization, blocks the enzymatic activity of NADPH oxidase complex, and attenuates intracellular ROS generation in microglia following LPS-stimulation.

siRNA knockdown of p22^{PHOX} and gp91^{PHOX} reduces the protective effects of mGluR5 activation

In order to confirm that inhibition of NADPH oxidase is a key mechanism through which CHPG attenuates microglial activation, we used siRNA to knockdown the expression of NADPH oxidase components p22^{PHOX} or gp91^{PHOX}. Western immunoblotting was performed to determine protein knockdown by targeted siRNA: p22^{PHOX} or gp91^{PHOX} protein expression was reduced by 52% and 60% respectively compared to control-siRNA transfected cell levels (Fig. 13Ai and ii). As mGluR5

receptor activation by CHPG pre-treatment significantly attenuates LPS-stimulated NO and TNF α release from BV2 microglia (Fig. 9A and 10C), these pro-inflammatory mediators were measured in control-, p22^{PHOX}- and gp91^{PHOX}-siRNA transfected cells to determine if NADPH oxidase knockdown alters the protective actions of CHPG. In BV2 microglia that expressed the scrambled control-siRNA, CHPG pre-treatment resulted in a $54.98 \pm 1.59\%$ reduction in LPS-stimulated NO release (Fig. 13B). In contrast, in BV2 microglia expressing p22^{PHOX}- or gp91^{PHOX}-siRNA, CHPG pre-treatment caused a $33.76 \pm 1.22\%$ and $28.73 \pm 2.22\%$ reduction, respectively. Similarly, CHPG pre-treatment in control-siRNA microglia resulted in a $54.52 \pm 2.82\%$ reduction in LPS-stimulated TNF α release (Fig. 13C), whereas CHPG treatment in p22^{PHOX}- or gp91^{PHOX}-siRNA microglia resulted in a $38.28 \pm 2.04\%$ and $34.73 \pm 3.88\%$ reduction in TNF α release, respectively. These data indicate that a reduction in NADPH oxidase membrane subunits p22^{PHOX} or gp91^{PHOX} by between 50 and 60% results in an approximate 38% loss of CHPG's effectiveness. These data further indicate that a key mechanism through which CHPG reduces microglial activation and the release of pro-inflammatory mediators is by directly inhibiting the NADPH oxidase complex.

mGluR5 stimulation also attenuates IFN γ -dependent microglial activation

In order to determine if CHPG's modulatory effects on microglia were unique for LPS-stimulation an alternative CNS immuno-activator was tested. The pro-inflammatory cytokine, IFN γ , stimulates microglial activity and participates in microglial-mediated neurotoxicity. BV2 microglia were pre-treated with CHPG (4mM) for 1 hr and subsequently stimulated with increasing concentrations of IFN γ (0.5 and 2ng/ml) for a further 24 hrs. IFN γ dose-dependently stimulated the production of NO (Fig. 14A ***p<0.001 versus control; ANOVA), while CHPG pre-treatment reduced IFN γ -stimulated NO production by more than 50% at both concentrations (+++p<0.001 versus IFN γ ; ANOVA). Similarly, IFN γ -stimulated microglia released increasing concentrations of TNF α (Fig. 14B ***p<0.001 versus control; ANOVA). In contrast, CHPG pre-treatment prevented IFN γ -stimulated TNF α release in microglia (+++p<0.001 versus IFN γ ; ANOVA), demonstrating that microglial activation induced by another physiologically relevant immuno-stimulator was significantly attenuated by mGluR5 activation. Taken together, the LPS and

IFN γ studies indicate that mGluR5 activation suppresses key pro-inflammatory signaling pathways that are involved in microglial-mediated neurodegeneration.

Example 3

Materials and Methods

5 Spinal cord injury

Contusion SCI was performed in adult male Sprague Dawley rats. Rats (275 – 325g) were anesthetized with sodium pentobarbital (67 mg/kg, I.P.) and moderate injury was induced by dropping a 10g weight from 25 mm onto an impounder positioned on the exposed spinal cord at vertebral level T9. Sham-injured rats
10 underwent laminectomy without weight-drop.

CHPG administration

Thirty minutes after contusion injury, an intrathecal catheter (P-100, 1.52 outside diameter) was inserted two segments below the injury site (spinal T11). The catheter was attached to an Alzet mini-osmotic pump (Model 2001, Alzet, Cupertino, CA), loaded with either CHPG (41.6 mM in 1% DMSO in saline; Tocris Biosciences,
15 Ellisville, MI) or vehicle (1% DMSO in 0.9% saline). The administration rate was 1 μ l of drug or vehicle per hr for 7 days (2.9 pmol of CHPG/day). A dose response study for inflammatory responses compared a 7 day infusion of CHPG at 10mM (in 1% DMSO in saline) to 41.6mM CHPG and vehicle. Pumps were weighed prior to
20 insertion and after removal to ensure function during implantation.

Functional assessment

The Basso-Beattie-Bresnahan (BBB) scale was used to assess neurological function (n = 15/group; Table 1).

Table 1:

Survival Time	Number per Group	Outcome Measures
24 hours	5 CHPG 41.6mM	Tumor necrosis factor- α enzyme-linked immunosorbent assay
	5 vehicle	
	3 sham	
72 hours	4 CHPG 41.6mM	Immunohistochemistry
	4 vehicle	
	2 sham	
7 days	4 CHPG 41.6mM	Immunohistochemistry
	4 CHPG 10mM	
	4 vehicle	
7 days	4 CHPG 4.16mM	Western blot
	4 vehicle	
	2 sham	
28 days	15 CHPG	Functional assessment
	41.6mM	
	15 vehicle	
	<i>Subgroups (randomly selected)</i>	
	5/group	Magnetic resonance imaging
	4/group	Immunohistochemistry
	4/group	Western blot

All functional scores were obtained at days 1, 7, 14, 21, and 28 by two individuals blinded to treatment. Data are presented as an average of raw score per group, as well as slope of the recovery curve from 1 to 28 days post-injury.

MRI analysis

At 28 days post-injury, a random sample of rats (n = 5/group) were chosen and underwent magnetic resonance imaging (MRI) using a 7 Tesla 20cm bore MRI (Bruker Biospin, Billerica, MA) with a 2D T2 weighted imaging protocol. The TR = 3640 msec, TE = 121 msec, MTX = 256 x 256. Hyperintense areas, which appeared as white regions in the normally gray spinal cord, on MRI images were assessed using Image J analysis software by a blinded investigator. Briefly, regions of interest were outlined and Image J analysis software was utilized to measure the area of the region. Areas were then used to extrapolate lesion volume throughout the injured cord.

Histology

The day after T2-weighted MRI imaging, a random sample of rats (n = 4/group) were anesthetized (100 mg/Kg sodium pentobarbital, I.P.) and intracardially perfused with 10% buffered formalin. To determine volume of cavitation and white matter sparing, every 20th section (20 μ m) of the 1 cm spinal cord block centered at the lesion epicenter, with a random starting section, was processed with a standard eriochrome cyanine R (Sigma, St. Louis, MO) staining protocol. Cavitation and spared white matter volume were assessed using unbiased stereology and the Stereologer Program (Systems Planning and Analysis, Alexandria, VA).

Immunohistochemistry

Standard fluorescent immunohistochemistry was performed on sections from animals obtained at 72 hours, 7 days or 28 days post-injury (n = 4/group; 28 day rats were randomly chosen). Anti-ED1 (Serotec, Raleigh, NC), OX42 (Serotec, Raleigh, NC), Iba-1 (Wako, Richmond, VA), anti-galectin-3 (Abcam, Cambridge, MA), gp91^{phox} (BD Transduction Laboratories, San Jose, CA), and anti-mGluR5 (Chemicon, Billerica, MA) were used as primary antibodies. Immunofluorescence was detected and quantified in 12 20 μ m sections, selected with a random start and consistent interslice distance, using confocal microscopy. The proportional area of tissue occupied by immunohistochemically stained cellular profiles within a defined target area (the lesion site and surrounding tissue) was measured using the Scion Image Analysis system using a method modified from that described by Popovich et al. (1997) J. Comp Neurol., 377:443-464).

Western blot

At 7 or 28 days post-injury, the 1 cm section of the spinal cord encompassing the lesion site was dissected from 4 rats/group. Rats from the 28 day group were randomly chosen. Tissue was homogenized and underwent Western blotting as described previously for p22^{phox} (Santa Cruz Biotechnologies, Santa Cruz, CA), ED1 (Serotec, Raleigh, NC), galectin-3 (Abcam, Cambridge, MA) and iNOS (BD Transduction Laboratories).

TNF α ELISA

At 24 hours post-injury, rats (n = 5/group + 3 Sham) were perfused with cooled saline and the 1 cm section of spinal cord encompassing the lesion site was

carefully dissected and frozen on dry ice. The concentration of TNF α was measured and ELISA was performed as per the manufacturer's instructions (BD Bioscience, San Jose, CA). Absorbance was read at 450 nm and values were corrected for protein concentration and expressed as pg/mg protein.

5 **Microglial cultures**

Microglial cells were obtained from postnatal day 2 Sprague Dawley rat pup spinal cords and cultured. After the initial incubation, the cells were shaken for 1 hour at 100 rpm and 37°C. Detached microglia were collected and replated at 2 x 10⁵ cells/ml into 96 well plates for proliferation and nitric oxide assays, or at 5 x 10⁵ cells/ml into Transwell® inserts (Fisher Scientific, Pittsburgh, PA) for co-culture assays.

Microglial proliferation

At 24 hours after application of the mGluR agonist CHPG and stimulation with LPS, proliferation of microglia in 96-well plates was assessed using the MTS assay (MTS tetrazolium compound; Cell Titer 96Aqueous One Solution®, Promega, Madison, WI), performed according to the manufacturer's protocols. Each treatment was performed in triplicate and the experiment was repeated 3 times.

Nitric Oxide production

Nitric oxide (NO) production was assayed at 24 hours after administration of LPS using the Griess Reagent Assay (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Each treatment was performed in triplicate and the experiment was repeated 3 times.

Neurotoxicity

Rat primary cortical neuronal cultures were derived from E18 rat cortices (Taconic, Germantown, NY). Microglia were stimulated with LPS for 24 hours, with or without pre-treatments, and washed in warmed media prior to insertion into 24-well plates containing neurons (neurons were at day 5 *in vitro*). Twenty-four hours later, microglia were removed and the neurons were fixed with 4% paraformaldehyde and stained with NeuN. The number of NeuN+ cells were counted in at least 10 areas around the 24 well plate. Each treatment was performed in triplicate and the experiment was repeated 3 times.

Statistical analysis

Quantitative data are presented as mean $\pm$ SEM. Lesion volume, functional data, Western blot and immunohistochemical data were obtained by an investigator blinded to treatment group. BBB scores were analyzed with two-way ANOVA and repeated measures. Remaining data were analyzed using Student's t-test or one-way ANOVA, where appropriate. All statistical tests were performed using the GraphPad Prism® Program, Version 3.02 for Windows® (GraphPad Software, Inc. San Diego, CA). A p value < 0.05 was considered statistically significant.

Results

CHPG treatment improves recovery after SCI

To test the neuroprotective effects of mGluR5 activation *in vivo*, adult male Sprague Dawley rats were subjected to moderate SCI and treated with the selective mGluR5 agonist CHPG or vehicle (7-day intrathecal infusion beginning at 30 minutes post-injury of 41.6mM CHPG or vehicle; 1 μ l/hr infusion rate; n=15/group). Function was assessed weekly, beginning at day 1 following injury, to determine recovery. By day 1 all animals had a score of 0 or 1, indicating complete or nearly complete loss of motor function (Fig. 15A). By day 14 post-injury, rats that received CHPG infusion had significantly improved BBB scores (12.3 $\pm$ 1.3; $p < 0.05$ with repeated measures ANOVA) compared with vehicle-treated rats (8.50 $\pm$ 0.9). This improvement remained through 28 days post-injury (12.4 $\pm$ 1.3 for CHPG *versus* 9.5 $\pm$ 0.7 for vehicle; $p < 0.05$ with repeated measures ANOVA). In terms of function, CHPG-treated rats showed coordinated walking steps, whereas vehicle-treated rats showed an ability to bear weight on their hindlimbs, but were unable to take walking steps.

The slope of the recovery curve was also assessed (Fig. 15B). CHPG treatment resulted in a significant increase in slope in comparison to vehicle treatment, indicating an increased rate of recovery.

mGluR5 activation reduces lesion volume after SCI

Rat spinal cords were imaged at day 28 post-injury using a T2-weighted MRI protocol. Analysis of hyperintense areas on resultant images demonstrated longitudinal lesions centered at T9 (Figs. 16A, B). Quantitation of hyperintense areas demonstrated a significant reduction in lesion volume with CHPG infusion ($p < 0.05$, student's t-test; Fig. 16C).

To determine if MRI-based lesion volumes reflected tissue cavitation, cavity volume was quantified in eriochrome-stained tissue sections (Fig. 17A). CHPG infusion resulted in a marked reduction in tissue cavity volume ($p < 0.05$, student's t-test; Fig. 17B). This reduction in tissue cavity was accompanied by a significant increase in the amount of spared white matter ($p < 0.05$, student's t-test; Fig. 17C).

Analysis of neuronal responses to injury and CHPG treatment revealed little evidence of neuronal apoptosis, as measured by double-labeling of NeuN and cleaved caspase-3 at the lesion site, rostral or caudal to the lesion epicenter. No significant difference in NeuN/cleaved-caspase-3 double-immunolabeling was observed between vehicle and CHPG-treated tissue.

mGluR5 activation reduces inflammation after SCI

To demonstrate that the effects of mGluR5 stimulation *in vivo* are mediated in part by suppression of inflammation, the expression of the activated microglia/macrophage marker Iba-1 was measured in the spinal cord 7 days after injury following vehicle or CHPG administration. Vehicle-treated tissue demonstrated strong Iba-1 staining throughout the spinal cord (Fig. 18A). In contrast, administration of CHPG markedly reduced SCI-induced Iba-1 staining (Fig. 18B, C). The reduction in Iba-1 staining was dependent on the dosage of CHPG applied, as increasing CHPG concentrations dose-dependently reduced Iba-1 immunolabeling (Fig. 18D), with a significant reduction in Iba-1 staining with 41.6mM CHPG treatment ($p < 0.05$, one-way ANOVA).

To further investigate the role of mGluR5 on inflammation, the presence of other inflammatory proteins and markers of microglial activation such as p22^{phox} and gp91^{phox}, components of the NADPH oxidase enzyme expressed in microglial cells, ED1, the common lysosomal marker for activated microglia and macrophages, Galectin-3 (MAC-2), a cell surface marker reportedly found only in microglia, inducible nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide, and the pro-inflammatory cytokine, TNF α were assessed after SCI. Spinal cord tissue homogenate was obtained at 24 hours after injury and TNF α protein expression was measured. Vehicle-treated tissue demonstrated a significant increase in TNF α protein content, in comparison to sham-injured tissue (Fig. 19). Treatment with CHPG significantly reduced the TNF α expression ($p < 0.05$, one-way ANOVA).

Tissue was obtained for Western blot at 7 and 28 days post-injury. At 7 and 28 days post-injury, CHPG treatment significantly reduced the expression of p22^{phox}, a membrane bound component of NADPH oxidase (Fig. 20A), when compared to vehicle-treated rats ($p < 0.05$, one-way ANOVA). Although there was a non-significant trend toward reduction in ED1 protein expression at 7 days, a marked reduction was observed at 28 days post-injury ($p < 0.05$, one-way ANOVA; Fig. 20B). Also at 28 days, CHPG infusion significantly reduced expression of Galectin-3/MAC2, a marker of microglial activation ($p < 0.05$, student's t-test; Fig. 20C), and iNOS, the enzyme responsible for production of NO ($p < 0.05$, student's t-test; Fig. 20D).

Furthermore, quantitation of immunohistochemical labelling for ED1 expression at 28 days demonstrated a significant reduction with CHPG treatment in comparison to vehicle treatment (student's t-test; Fig. 21).

mGluR5 activation reduces gp91^{phox} expression at 28 days post-SCI. Immunolabeling for gp91^{phox} was performed at 72 hours and 28 days post-injury in vehicle and CHPG-treated tissue. Analysis of immunolabeling for gp91^{phox}, the membrane bound catalytic subunit of NADPH oxidase, shows that there was no significant difference between vehicle and treated tissue at 72 hours. However, a significant reduction in gp91^{phox} staining was observed at 28 days with CHPG treatment ($p < 0.05$, one-way ANOVA; Fig. 22).

mGluR5 is expressed on microglia after SCI

To investigate whether microglia express mGluR5 *in vivo*, double immunohistochemical labelling was performed with mGluR5 and microglial markers. In injured spinal cord tissue, mGluR5 co-labelled with markers specific for microglia in the CNS, galectin-3⁴⁴ and OX42 (CD11b). This double-labelling was observed in the lesioned area at both the lesion epicenter and in the periphery. Negative controls, in which the primary antibodies were not included, failed to show the same labelling pattern.

mGluR5 stimulation reduces spinal cord microglial activation

As microglia demonstrate regional heterogeneity within the CNS, microglia were cultured from rat spinal cord to determine if spinal cord microglia are responsive to mGluR5 activation. These cells were treated with lipopolysaccharide (LPS;

100ng/ml) with and without 1 hour CHPG (100 μ M) pre-treatment. LPS stimulation of spinal cord microglia for 24 hours resulted in a marked increase in NO production and proliferation ($p<0.001$, one-way ANOVA; Fig. 23A, B). These increases were significantly reduced by CHPG treatment ($p<0.05$, one-way ANOVA; Fig. 23A, B), demonstrating that activation of mGluR5 on spinal cord microglia attenuates microglial reactivity.

mGluR5 stimulation reduces microglial-induced neurotoxicity

In order to determine if mGluR5 plays a role in microglial-induced neurotoxicity, cultured spinal cord microglia were cultured in transwell plates and stimulated with LPS (100ng/ml) with or without CHPG (100 μ M) pre-treatment. Twenty-four hours after stimulation with LPS, transwell plates were transferred into wells containing neurons. Stimulation of microglia with LPS prior to co-culture significantly decreased the number of NeuN+ neurons at 24 hours after co-culture ($p<0.05$, one-way ANOVA; Fig. 24). Pre-treatment of microglia with CHPG resulted in a significant increase in NeuN+ cells ($p<0.05$, one-way ANOVA). In order to eliminate the possibility of a direct effect of CHPG on neurons in this study, microglia were washed prior to addition to neurons. Neither LPS nor CHPG had any direct effect on neurons when added without microglia. Addition of the selective mGluR5 antagonist MTEP (100 μ M) to microglia prior to CHPG pre-treatment reversed the protective effect of CHPG, reducing neuronal viability ($p<0.05$; Fig. 24), indicating that CHPG acts through the mGluR5 receptor.

Example 4

CHPG treatment at 30 minutes post injury

A traumatic brain injury was produced in mice. CHPG was administered 30 minutes after traumatic brain injury. Function was assessed using beam walk (for hindlimb function) and Morris Water Maze (for cognitive function). As shown in Fig. 25A, CHPG treatment resulted in a significant reduction in the number of errors in the beam walk test. As shown in Fig. 25B, CHPG significantly improved the performance on the water maze test, reducing the latency to find the hidden platform in comparison to the vehicle-treated mice.

At 21 days post-injury, lesion volume was assessed using T2 weighted MRI. As shown in Fig. 26, CHPG administration at 30 minutes post-injury resulted in a significant reduction in lesion volume.

Example 5

5 CHPG treatment at 28 days post injury

A traumatic brain injury was produced mice. CHPG (10mM, 5ul, ICV) or vehicle (5% DMSO in saline) was administered at 28 days post-injury. Prior to treatment as well as at 2 and 3 months post-injury, motor function was assessed using the beam walk test. As shown in Fig. 27, mice that received CHPG demonstrated a significant increase in the percent of successful trials in the beam walk test, as compared to the vehicle treated mice. $P < 0.05$ at 2 and 3 months post-injury.

Lesion volume was assessed in mice ($n = 5/\text{group}$) at 1 month (prior to treatment), 2 months and 3 months (post-treatment) post-injury. CHPG was applied at 1 month post-injury. As shown in Fig. 28, lesion volume was measured using T2 weighted MRI imaging, and showed a significant reduction in CHPG-treated mice at 2 and 3 months post-injury.

Moreover, there was a reduction of lesion size observed in the CHPG treated brain. Fig. 29 shows representative T2 weighted MRI images at 3 months post-injury (2 months after administration of CHPG or vehicle). Hyperintense regions indicate areas of lesion in the left cortex.

WHAT IS CLAIMED IS:

1. A method of reducing or preventing neuroinflammation or neurotoxicity in a subject, comprising administering to the subject an mGluR5 agonist.
2. The method of claim 1, wherein the mGluR5 agonist is selected from the group consisting of a small molecule or a polypeptide.
3. The method of claim 1, wherein the mGluR5 agonist is administered systemically to the subject.
4. The method of claim 1, wherein the mGluR5 agonist is administered intraventricularly to the subject.
5. The method of claim 1, wherein the mGluR5 agonist is administered into the central nervous system of the subject.
6. The method of claim 1, wherein the subject has an central nervous system injury.
7. The method of claim 6, wherein the central nervous system injury is a spinal cord injury.
8. The method of claim 6, wherein the central nervous system injury is a stroke.
9. The method of claim 6, wherein the central nervous system injury is a head injury.
10. The method of claim 6, wherein the central nervous system injury is an acute injury.
11. The method of claim 6, wherein the central nervous system injury is a chronic injury.
12. The method of claim 1, wherein the subject has or is suspected of having a neurodegenerative disorder.
13. The method of claim 12, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's Disease, Parkinson's Disease, and multiple sclerosis.
14. A method of reducing activation of a microglial cell comprising contacting the microglia cell with an mGluR5 agonist.

15. The method of claim 14, wherein the contacting step is performed *in vitro*.
16. A method of treating a subject having a nervous system disease or injury, comprising administering to the subject having the nervous system disease or injury an mGluR5 agonist.
17. The method of claim 16, wherein the nervous system injury is selected from the group consisting of a stroke, traumatic brain injury, and spinal cord injury.
18. The method of claim 17, wherein the mGluR5 agonist is administered on the day of the injury.
19. The method of claim 17, wherein the mGluR5 agonist is administered after the day of the injury.
20. The method of claim 16, wherein the nervous system disease is selected from the group consisting of Alzheimer's Disease, Parkinson's Disease and multiple sclerosis.
21. A method of screening for an agent that reduces or prevents neuroinflammation or neurotoxicity comprising the steps of
 - (a) contacting microglia with the agent to be screened,
 - (b) stimulating microglia with an inflammatory stimulus, and
 - (c) detecting the level of NO, ROS, TNF α , or microglial proliferation, with a reduced level of NO, ROS, TNF α , or microglial proliferation compared to inflammatory stimulus treatment alone indicating the agent reduces or prevents neuroinflammation or neurotoxicity.

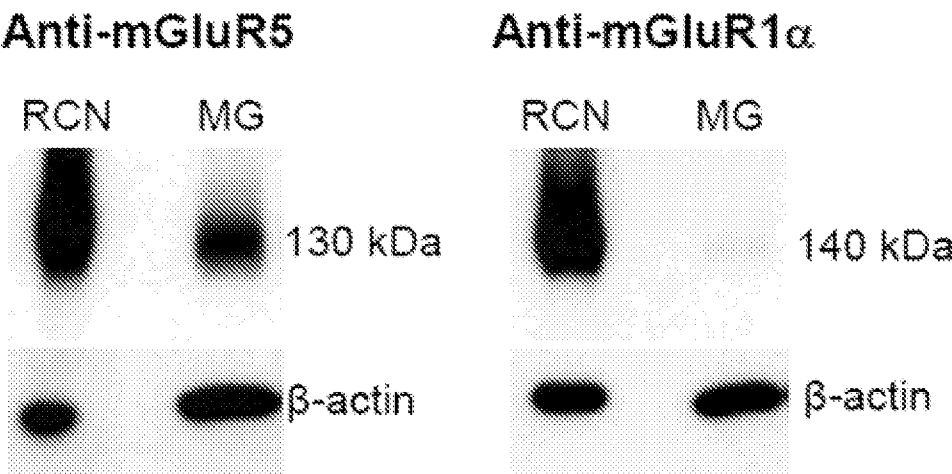


FIGURE 1

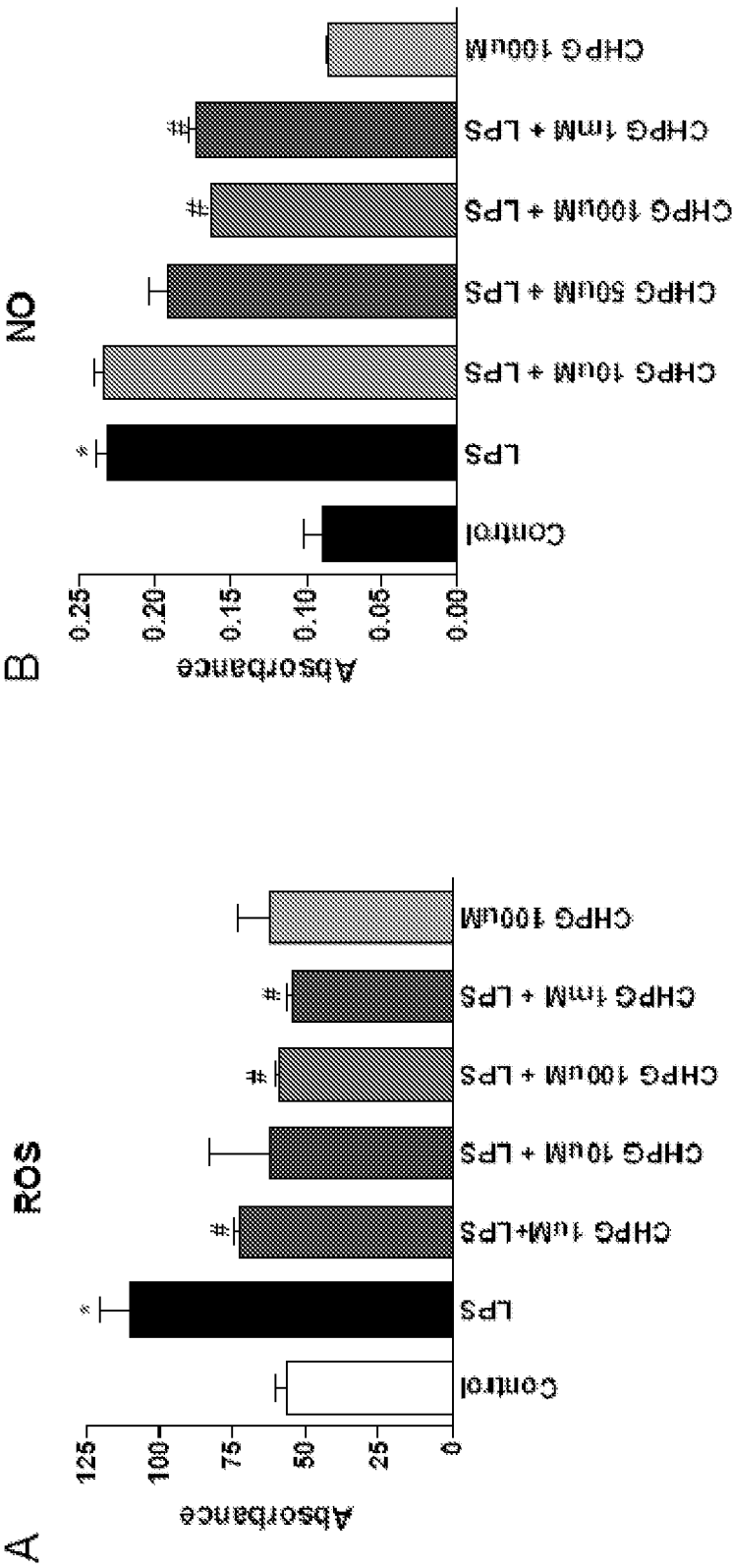


FIGURE 2

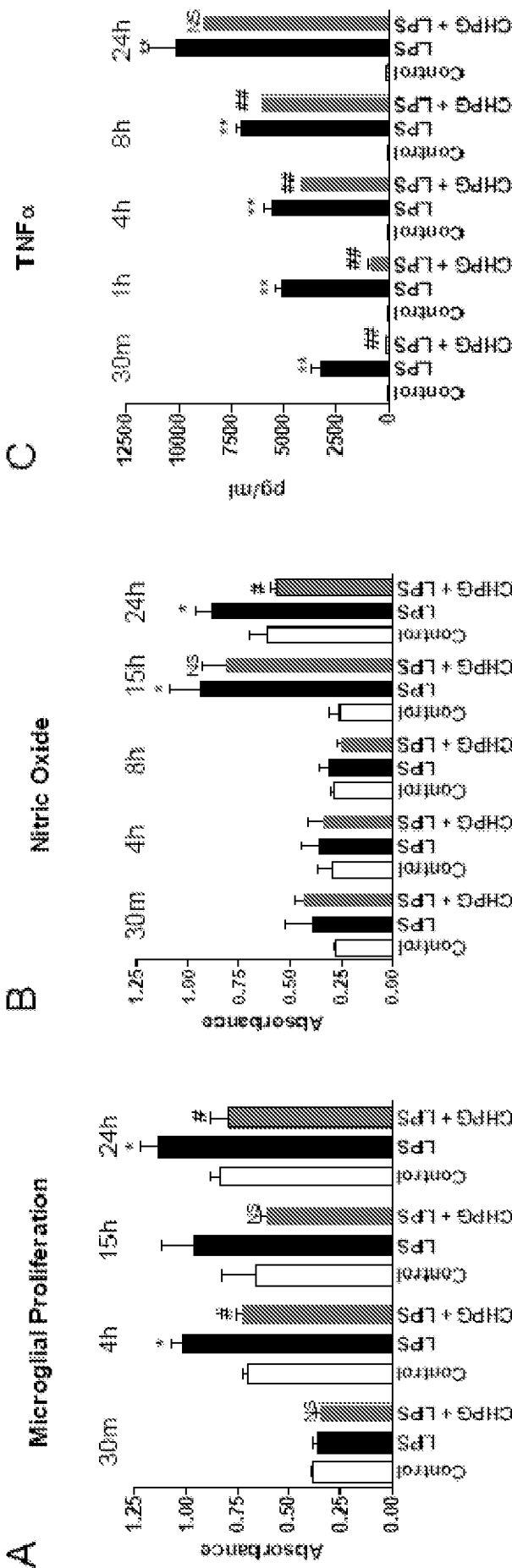


FIGURE 3

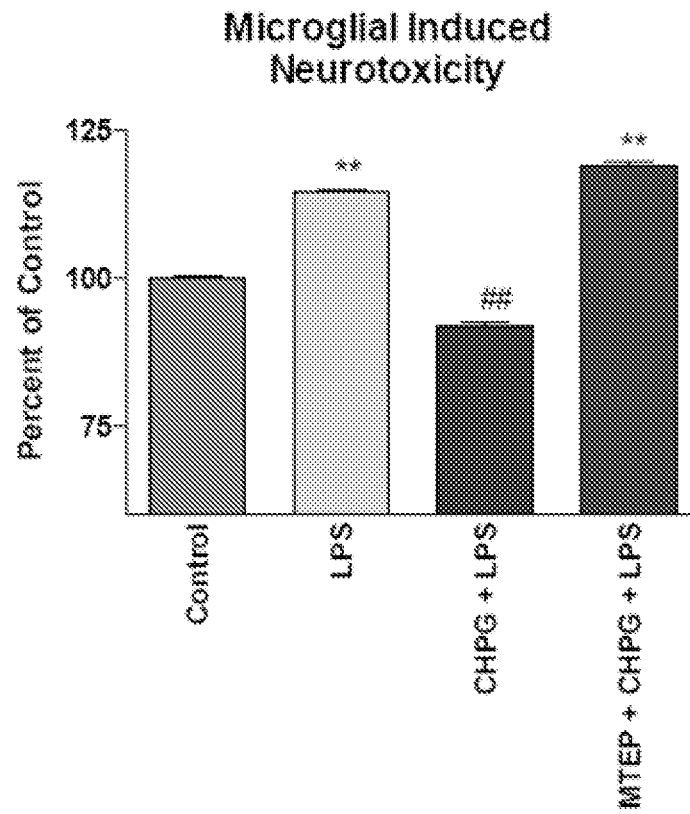


FIGURE 4

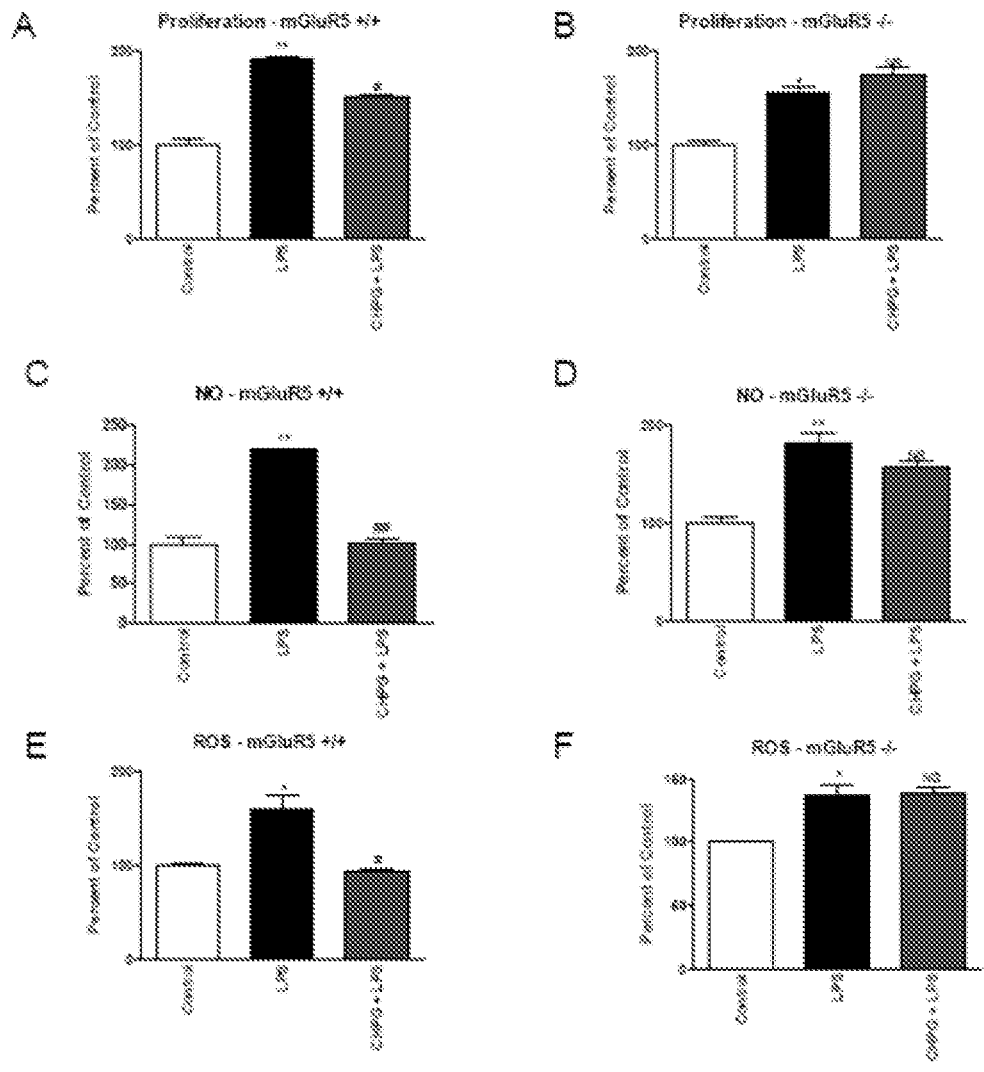


FIGURE 5

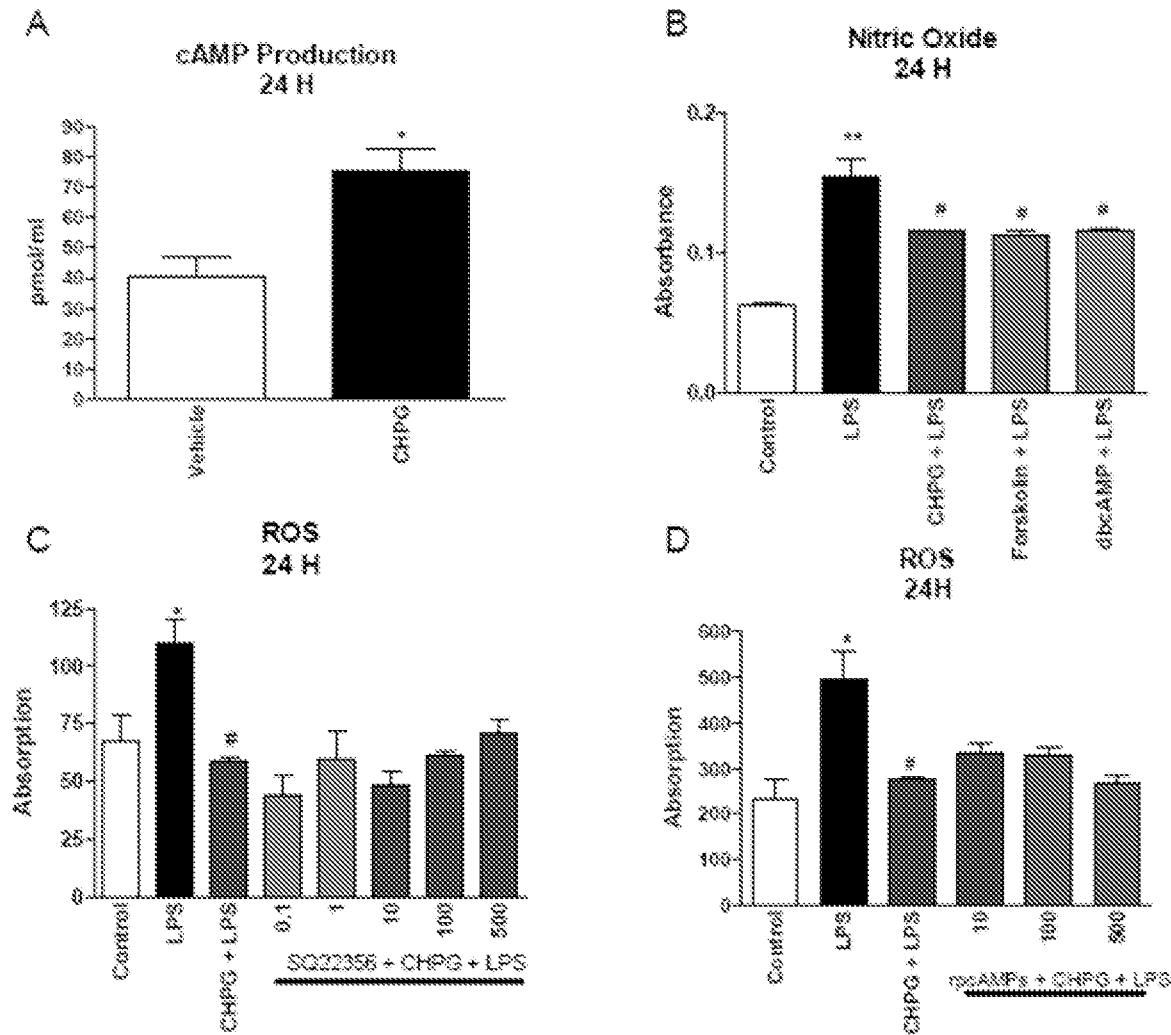


FIGURE 6

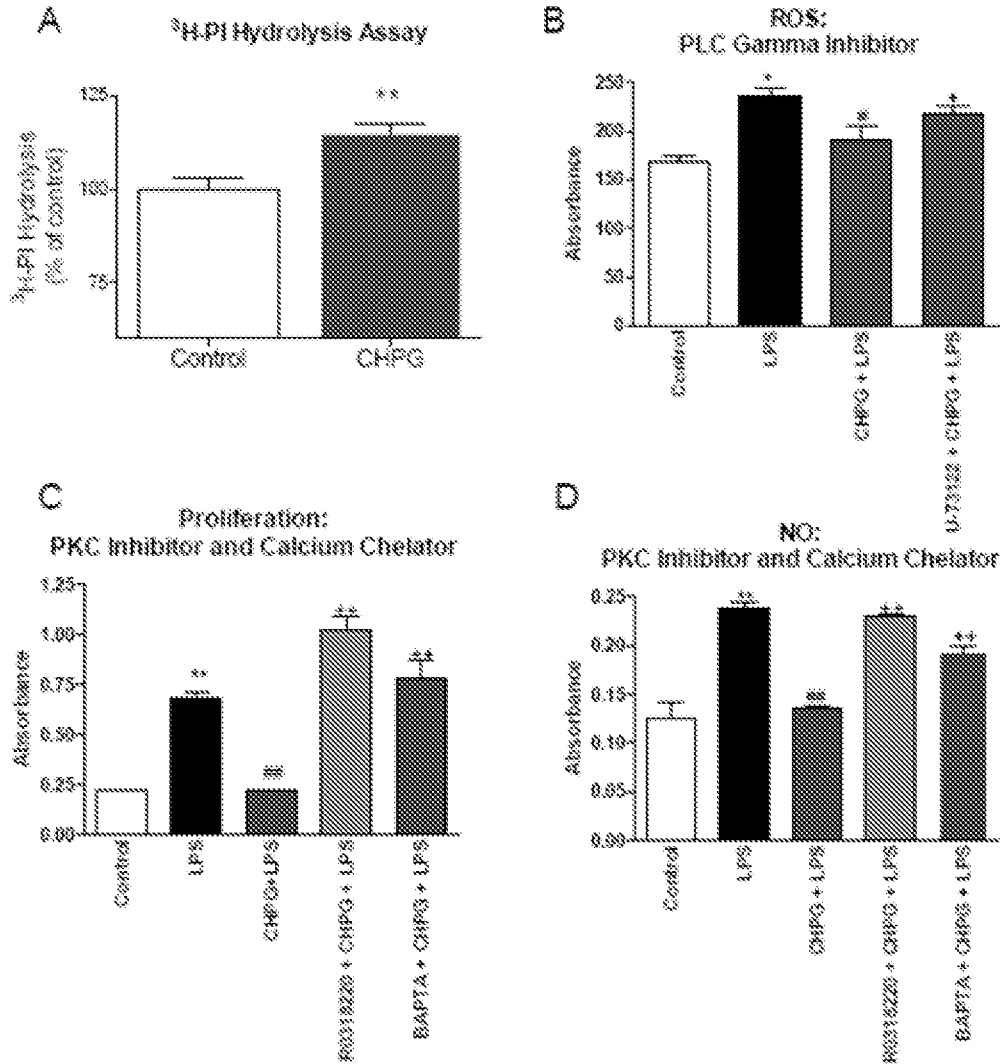


FIGURE 7

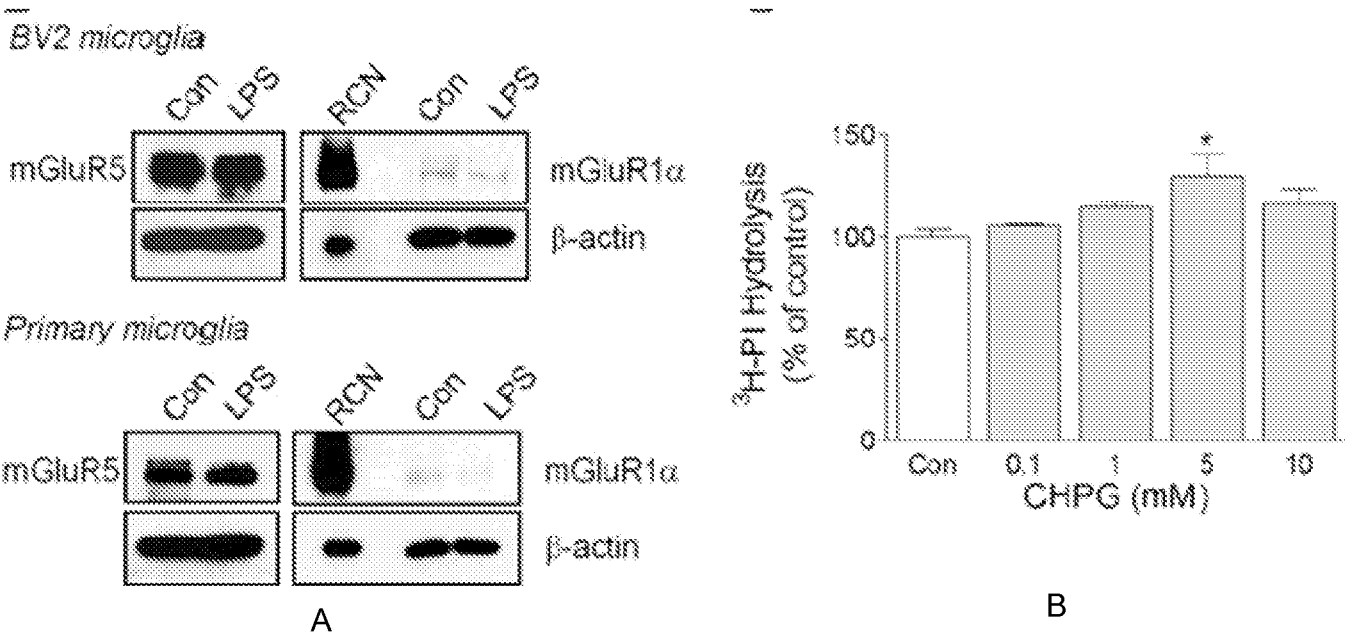


FIGURE 8

mGluR5 and NADPH Oxidase

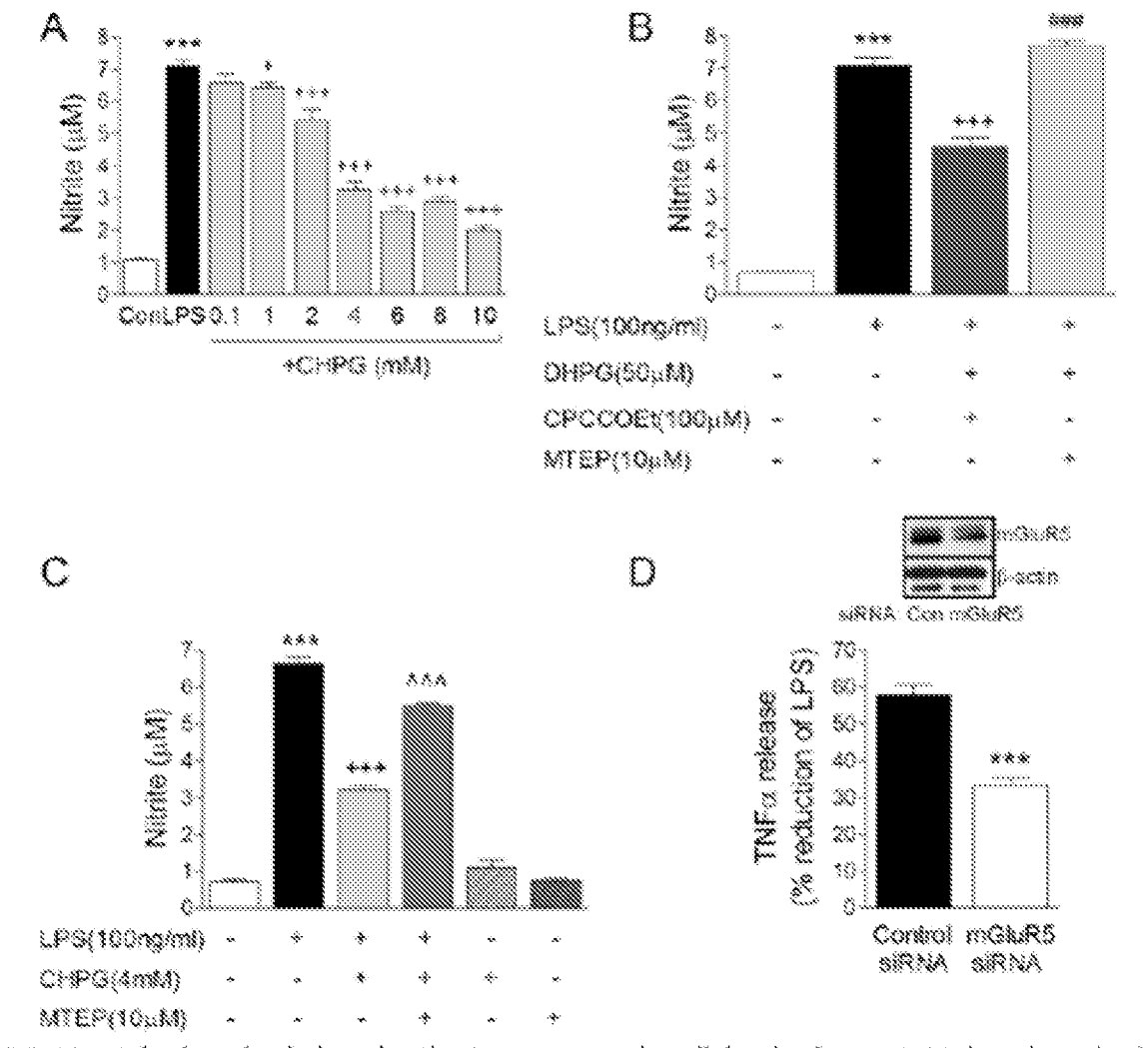


FIGURE 9

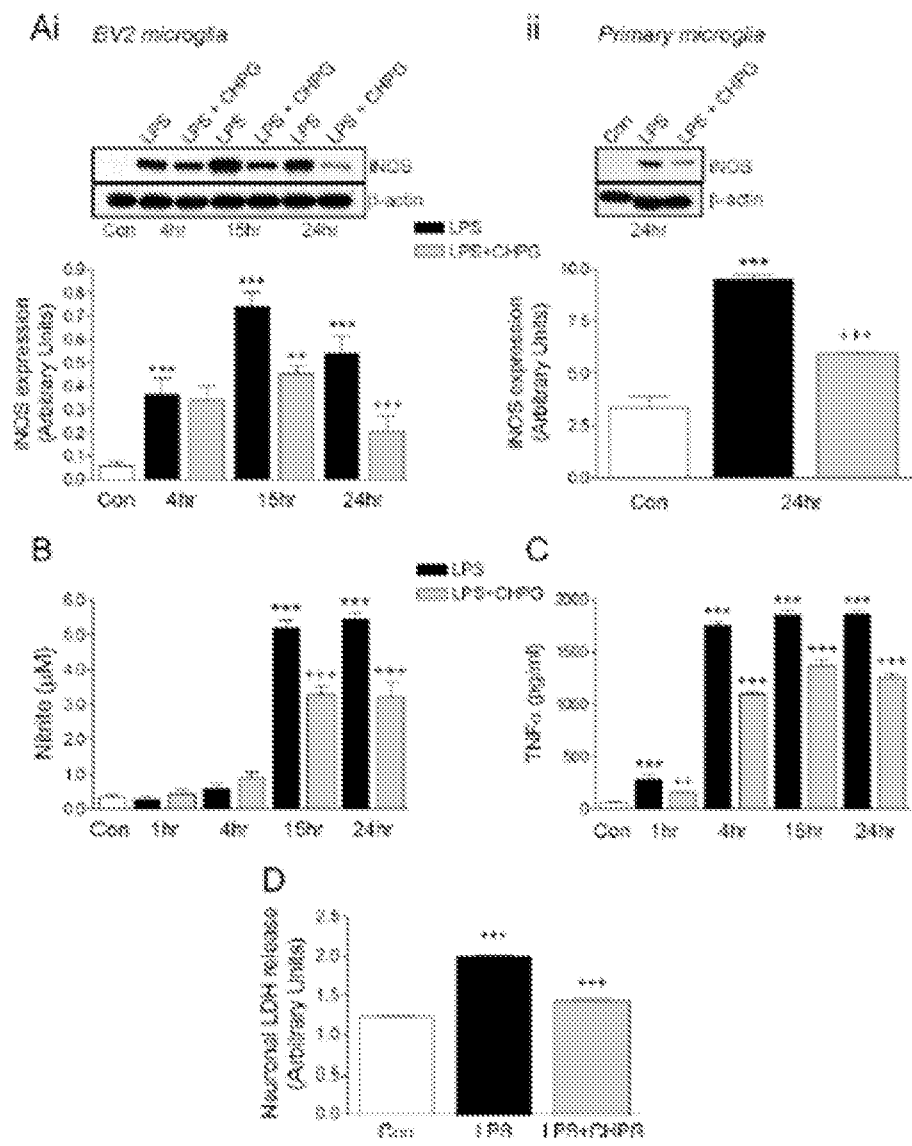


FIGURE 10

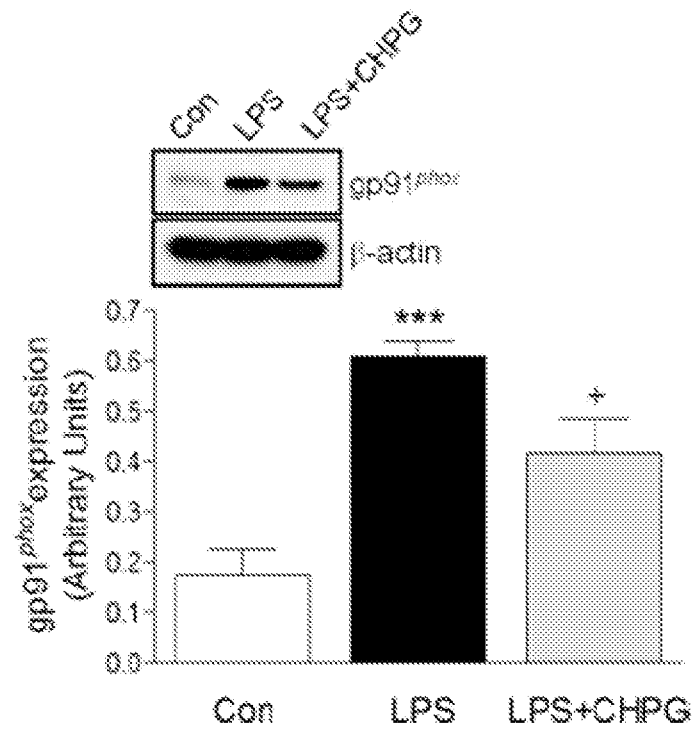


FIGURE 11

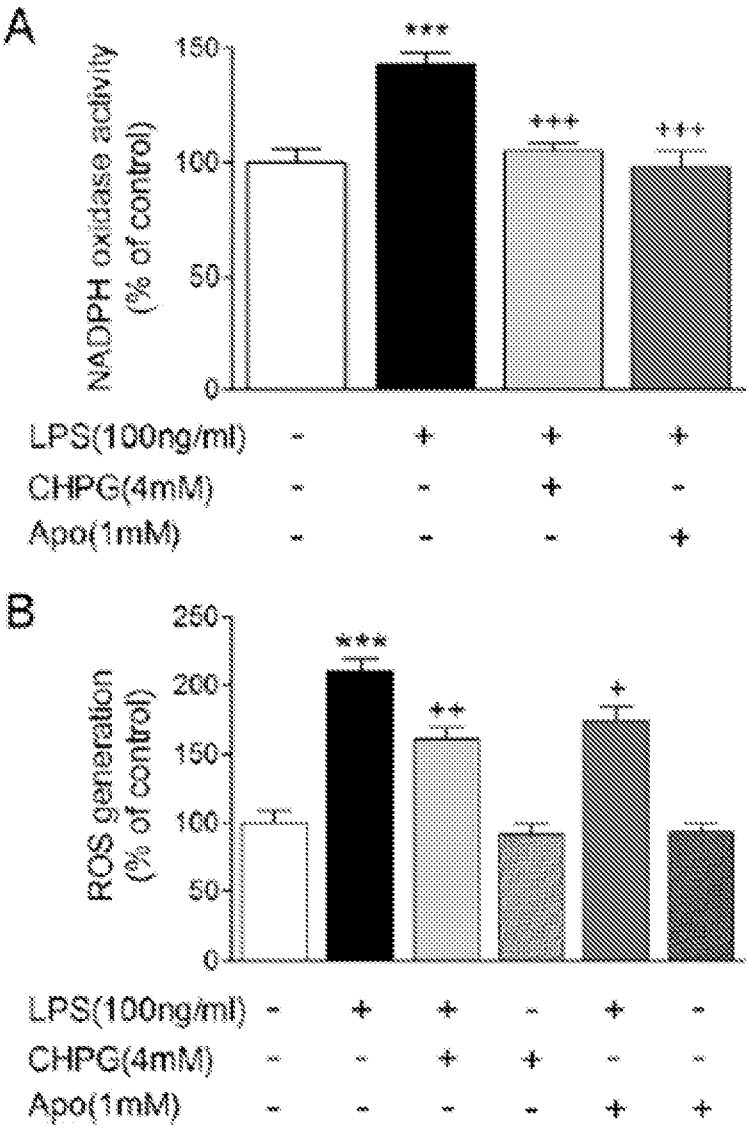


FIGURE 12

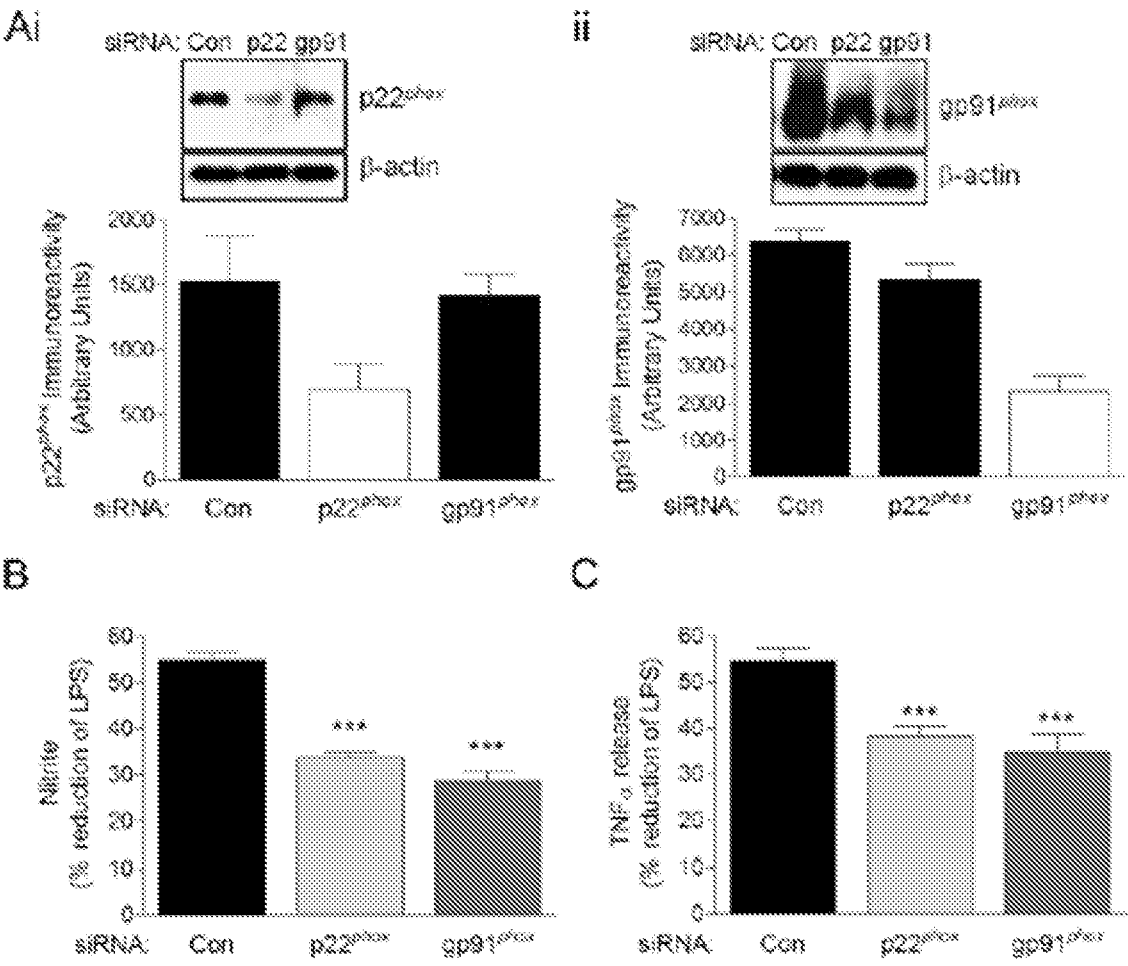


FIGURE 13

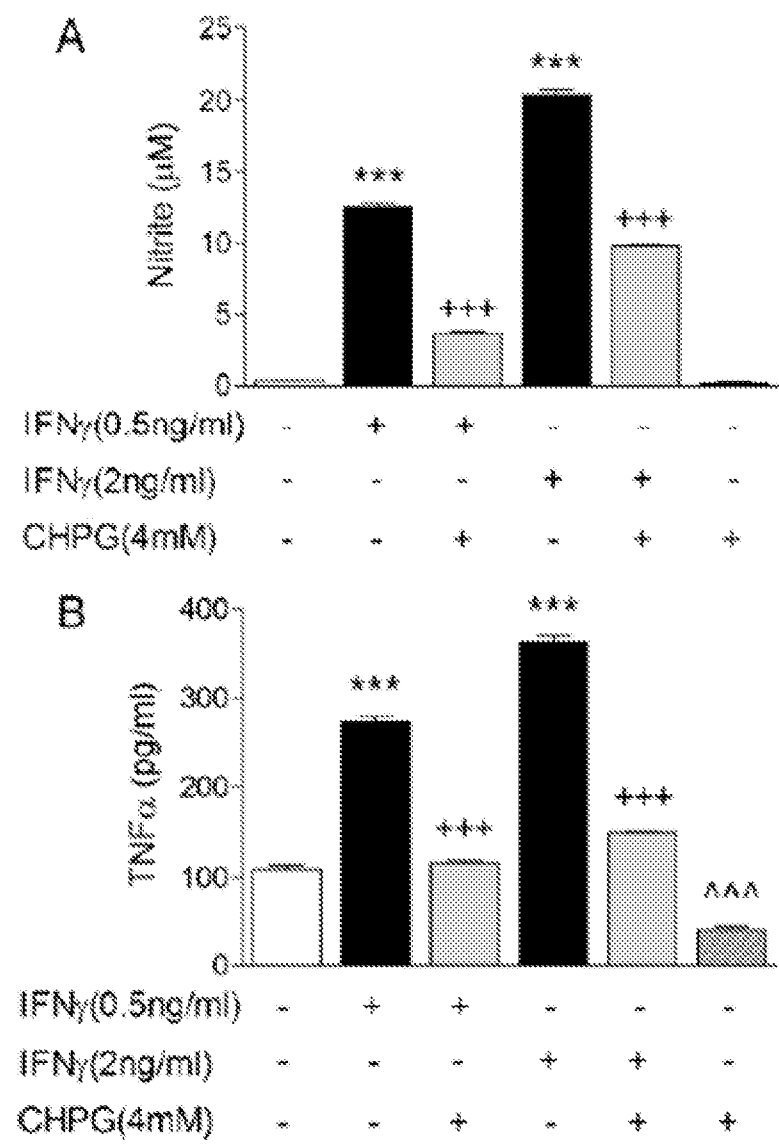


FIGURE 14

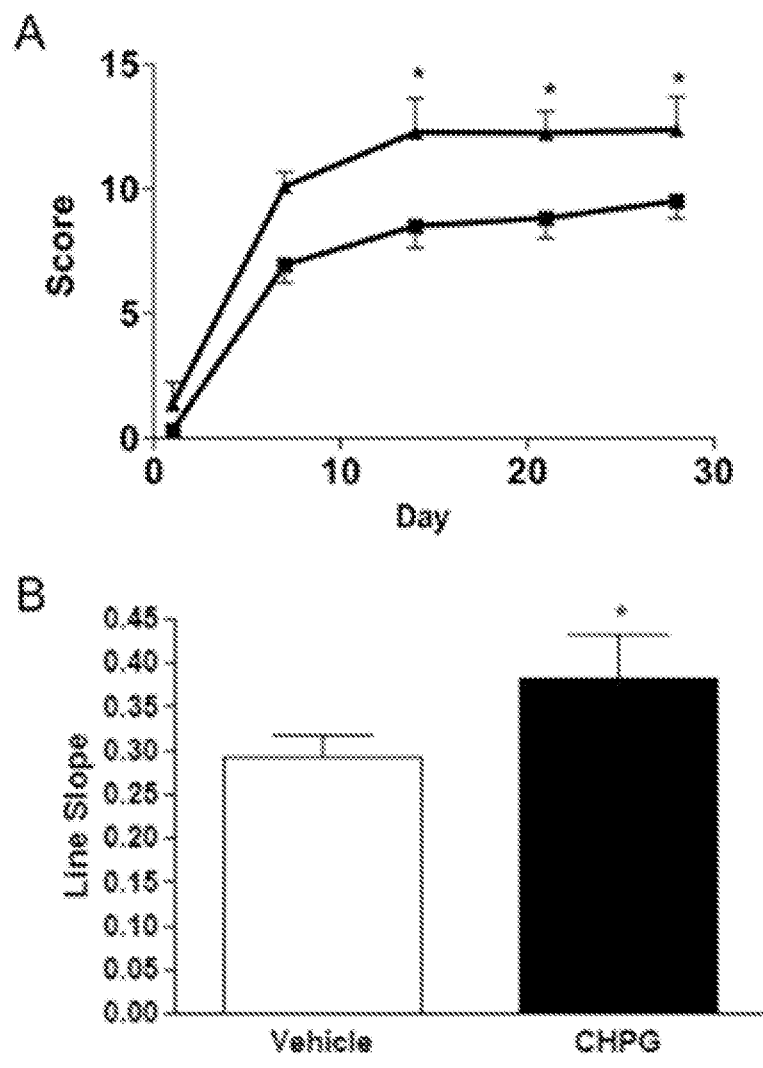


FIGURE 15

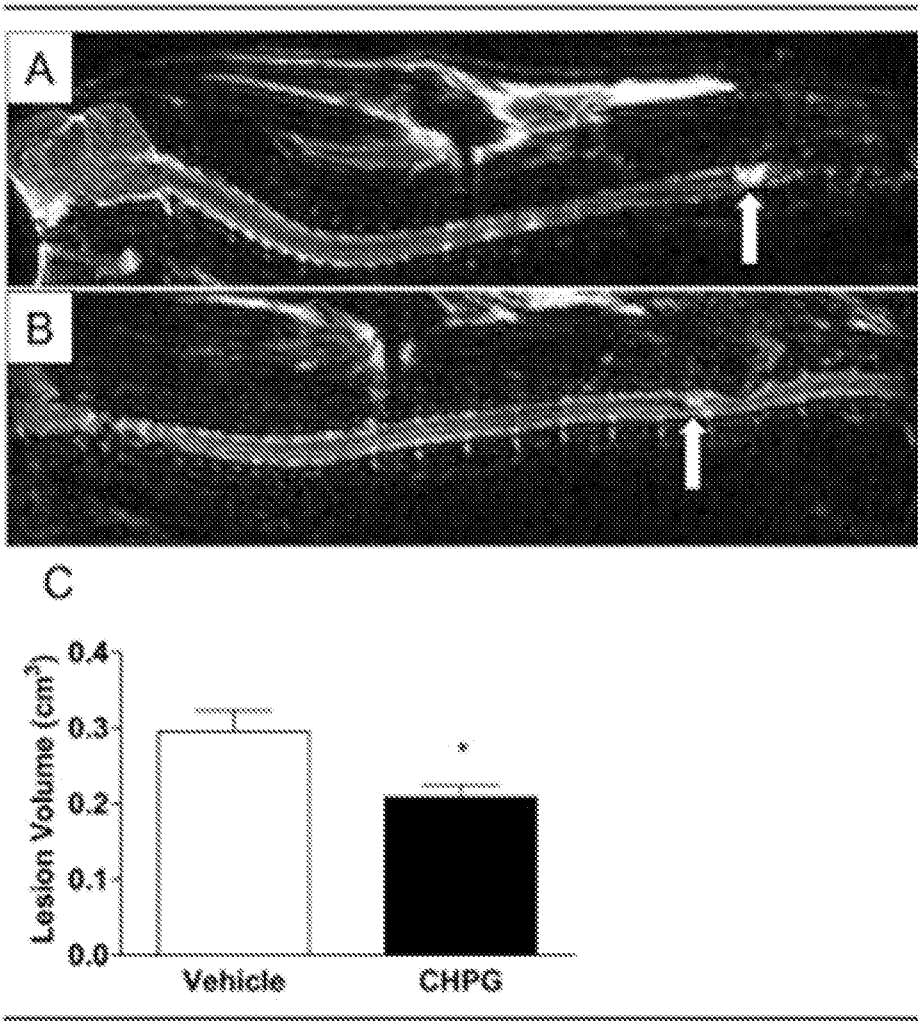


FIGURE 16

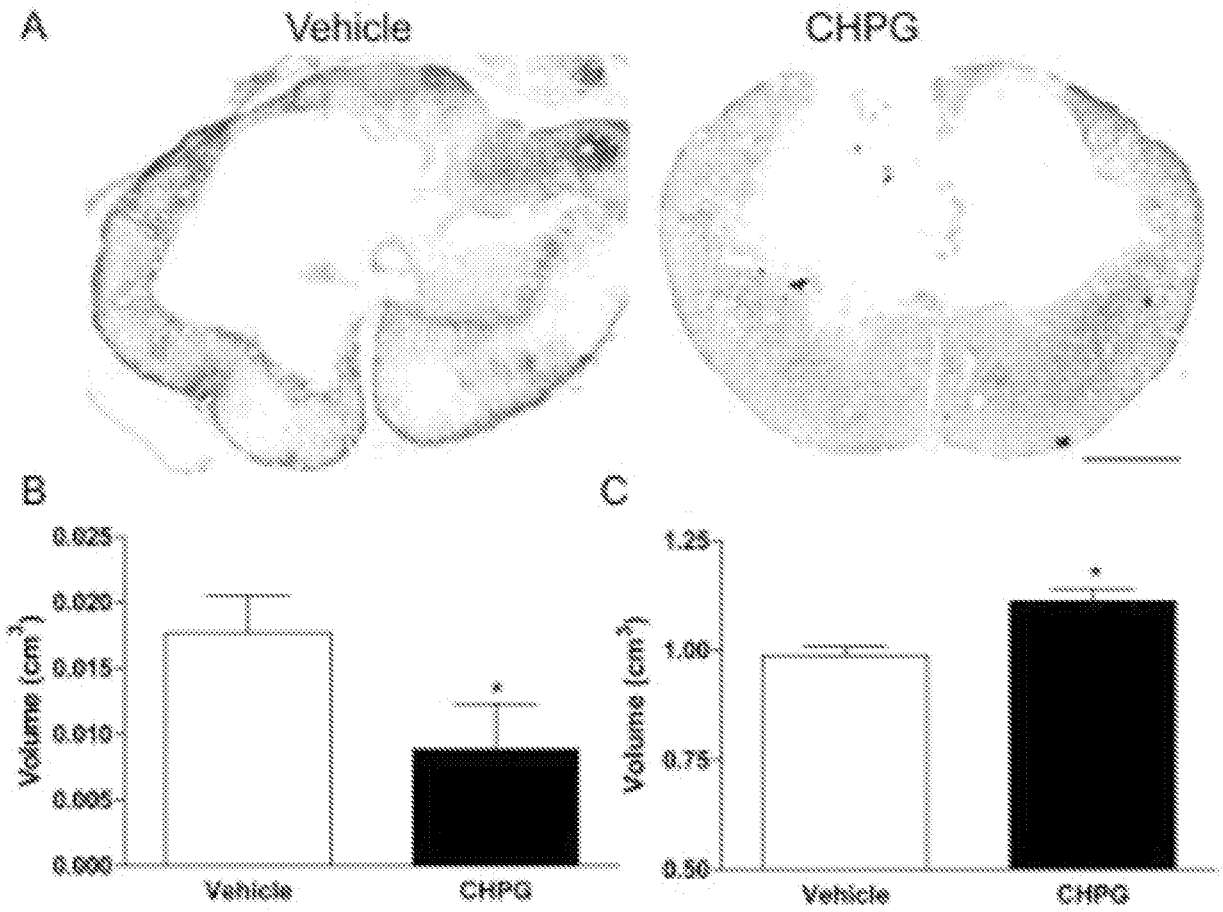


FIGURE 17

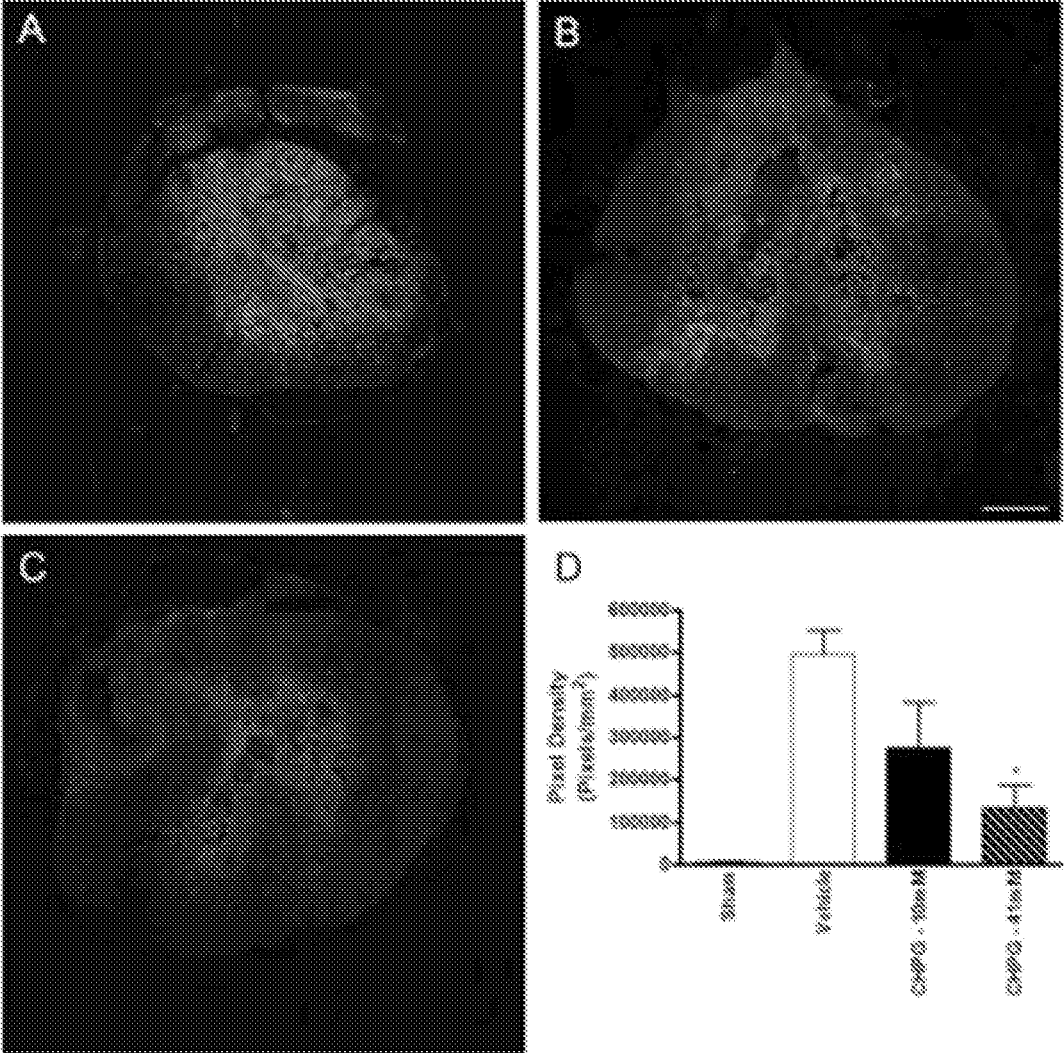


FIGURE 18

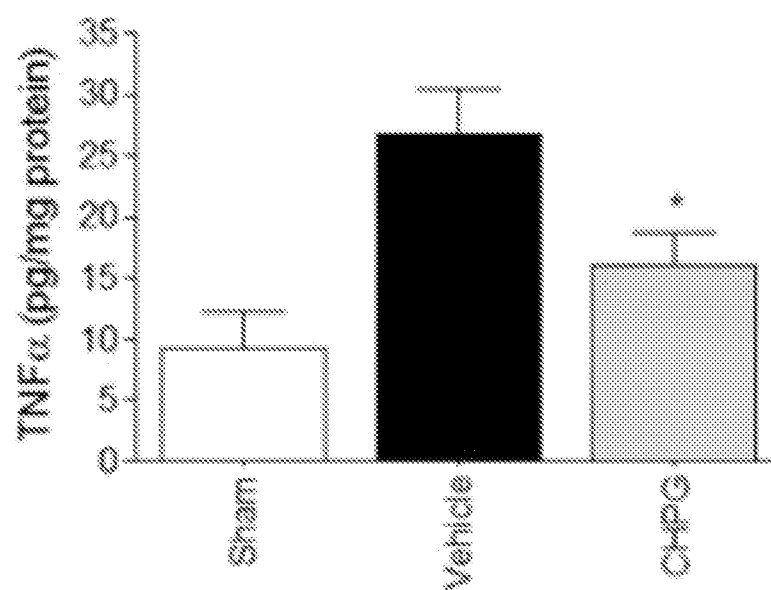


FIGURE 19

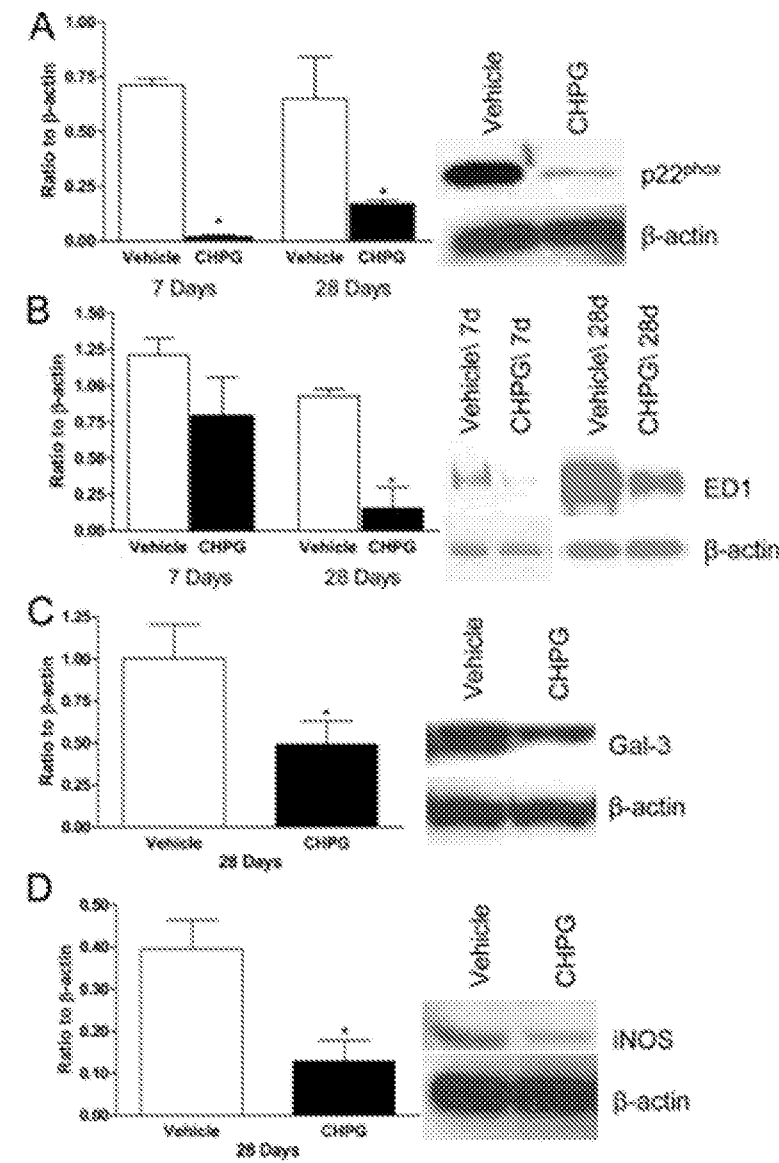


FIGURE 20

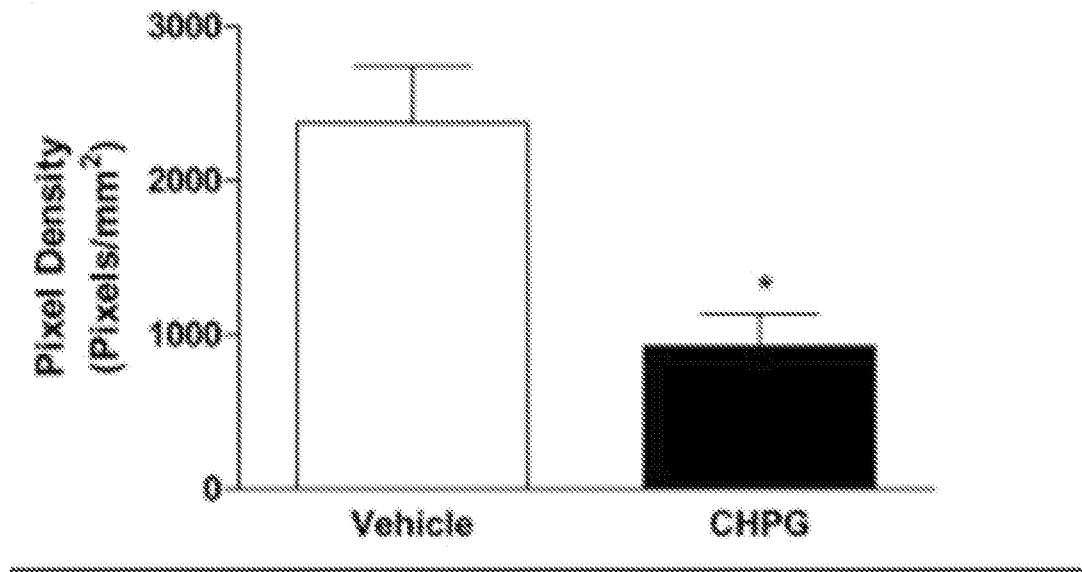


FIGURE 21

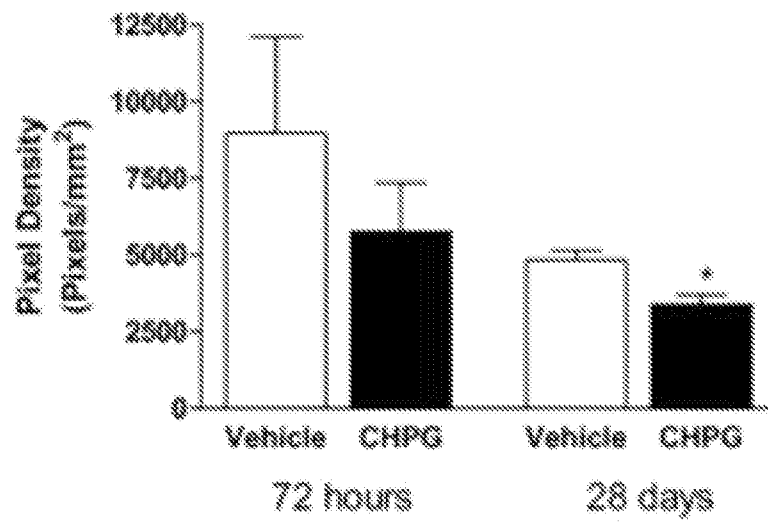


FIGURE 22

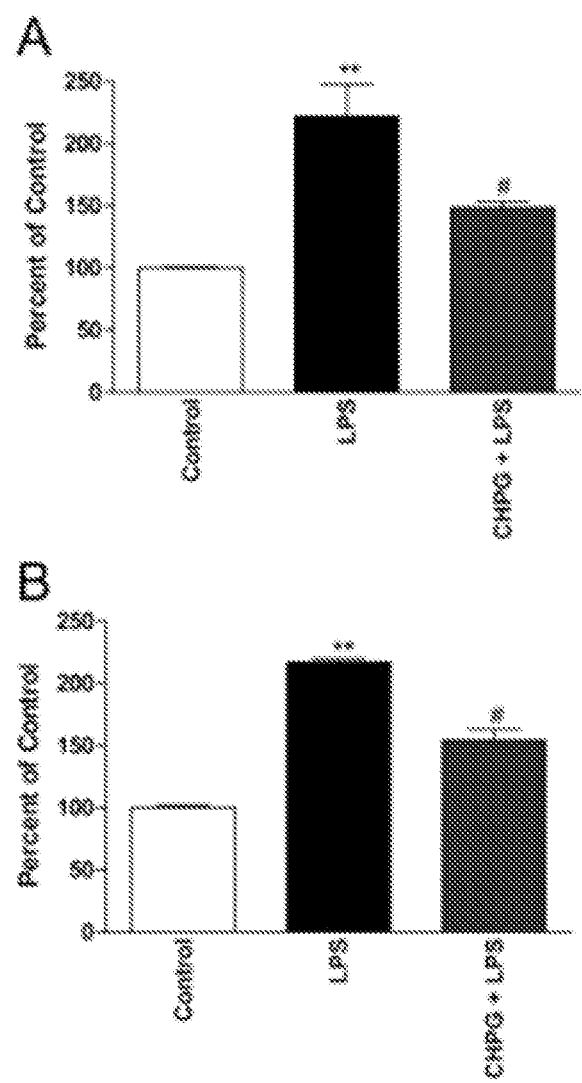


FIGURE 23

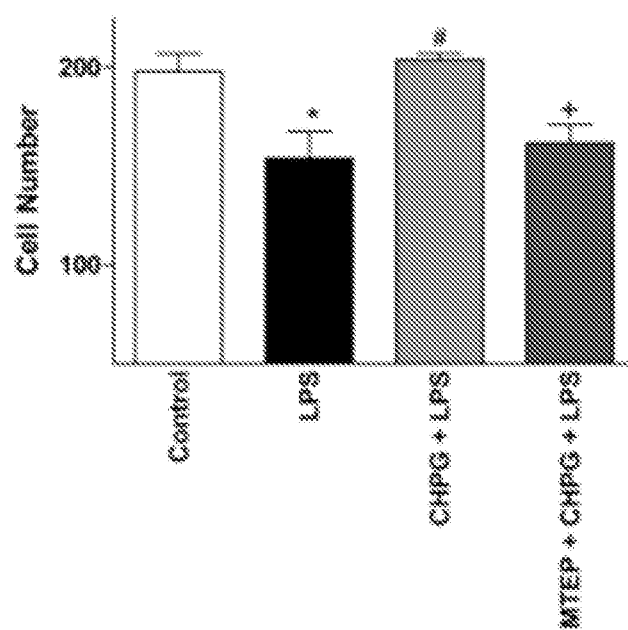


FIGURE 24

CHPG In Vivo:
(10mM, 5ul, 30 min after CCI)

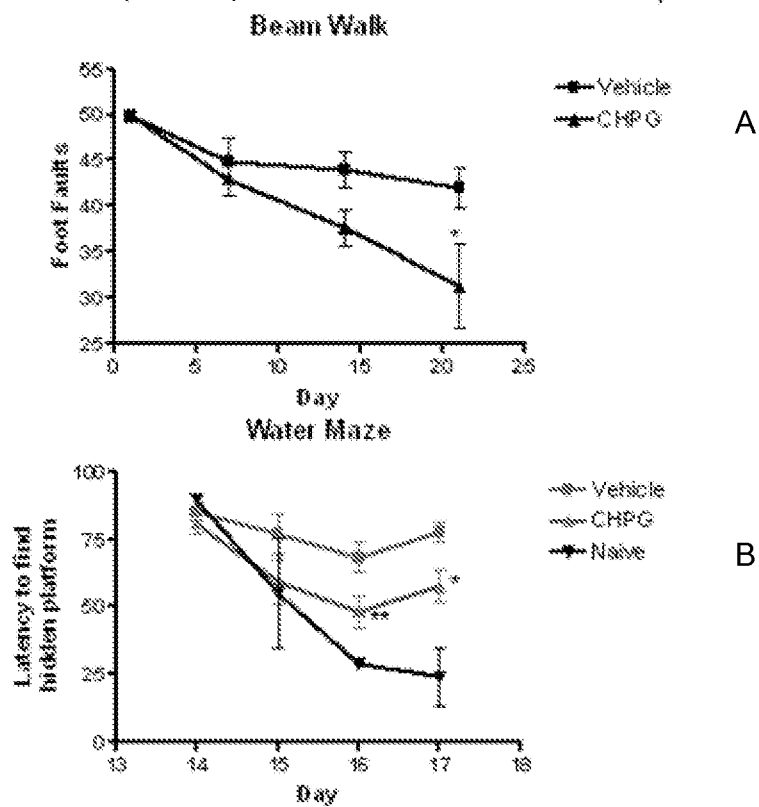


FIGURE 25

CHPG + CCI

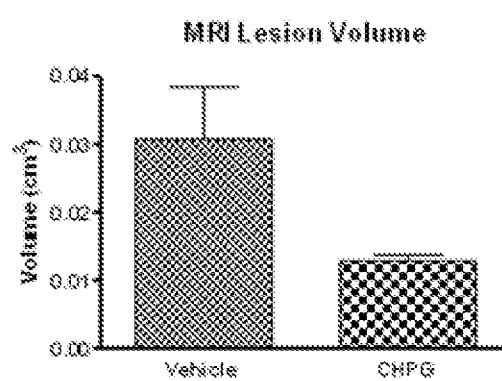


FIGURE 26

Function: Chronic CCI

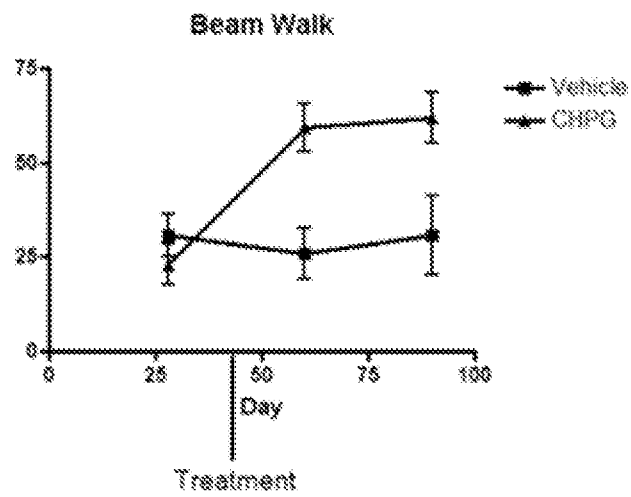


FIGURE 27

Chronic Study: MRI

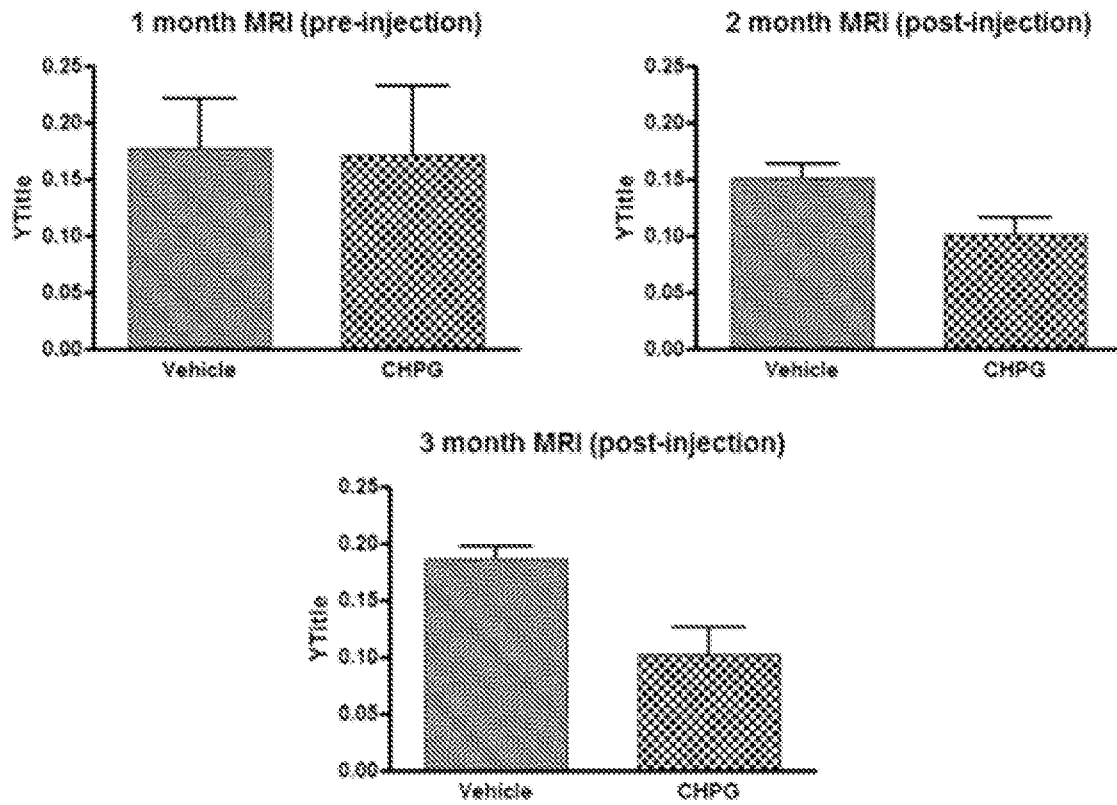


FIGURE 28

Representative MRI: 3 Months Post-Injury

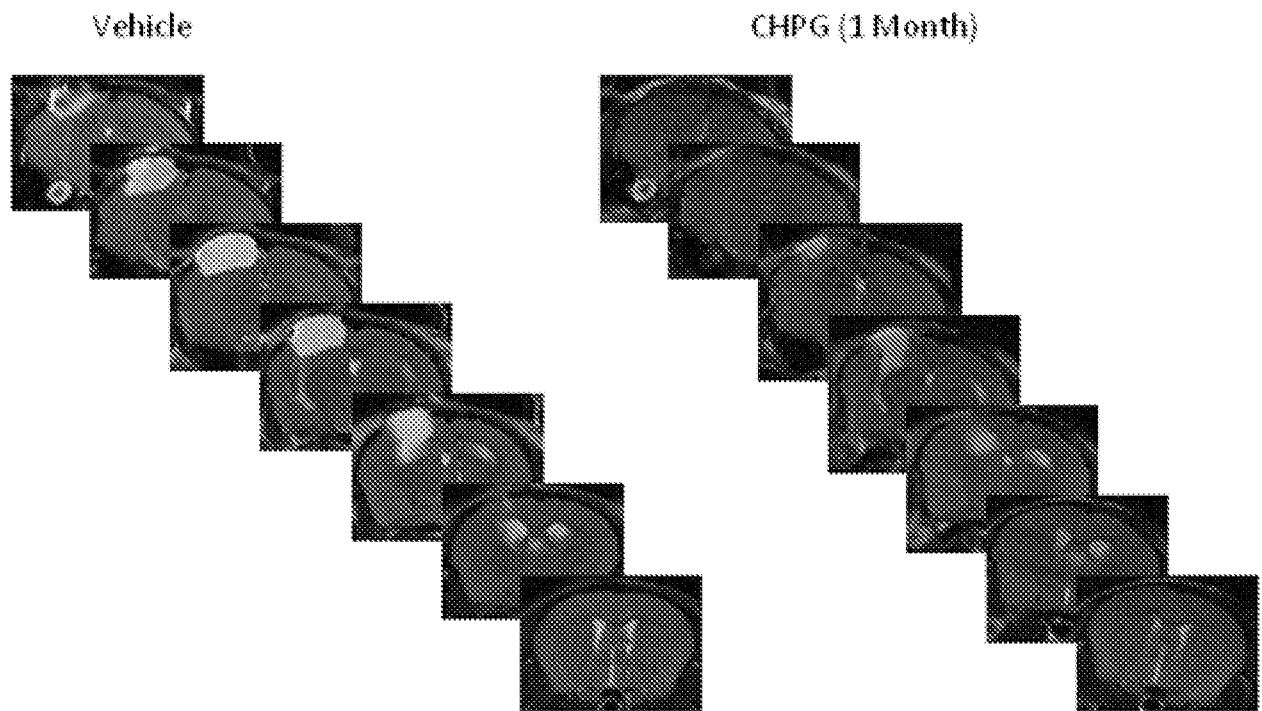


FIGURE 29