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NAAG Peptidase Inhibitor Improves Motor Function and Reduces Cognitive Dysfunction in a Model of TBI with Secondary Hypoxia

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Abstract

Immediately following traumatic brain injury (TBI) and TBI with hypoxia, there is a rapid and pathophysiological increase in extracellular glutamate, subsequent neuronal damage and ultimately diminished motor and cognitive function. *N*-acetyl-aspartyl glutamate (NAAG), a prevalent neuropeptide in the CNS, is co-released with glutamate, binds the presynaptic mGluR3 (group II metabotropic glutamate receptor) and suppresses glutamate release. However, the catalytic enzyme glutamate carboxypeptidase II (GCPII) rapidly hydrolyzes NAAG into NAA and glutamate. Inhibition of the GCPII enzyme with NAAG peptidase inhibitors reduces the concentration of glutamate both by increasing the duration of NAAG activity on mGluR3 and by reducing degradation into NAA and glutamate resulting in reduced cell death in models of TBI and TBI with hypoxia. In the following study, rats were administered the NAAG peptidase inhibitor PGI-02776 (10 mg/kg) 30 min following TBI combined with a hypoxic second insult. Over the two weeks following injury, PGI-02776 treated rats had significantly improved motor function as measured by increased duration on the rota-rod and a trend toward improved performance on the beam walk. Furthermore, two weeks post-injury, PGI-02776-treated animals had a significant decrease in latency to find the target platform in the Morris water maze as compared to vehicle-treated animals. These findings demonstrate that the application of NAAG peptidase inhibitors can reduce the deleterious motor and cognitive effects of TBI combined with a second hypoxic insult in the weeks following injury.

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Keywords

Traumatic brain injury (TBI); Hypoxia; Excitotoxicity; *N*-acetylaspartylglutamate (NAAG); Behavior; Pre-clinical

1. Introduction

In the United States, there are an estimated 1.7 million persons who sustain a traumatic brain injury (TBI) annually resulting in over 275,000 hospitalizations and 52,000 deaths (Faul et al., 2010). One of the hallmark pathologies in TBI patients is an excessive accumulation of extracellular glutamate (Brown et al., 1998; Chamoun et al., 2010; Koura et al., 1998; Vespa et al., 1998) that is correlated with lower Glasgow outcome scores at 6-months following injury (Koura et al., 1998). Similar to what is observed in patients, experimental models of TBI cause excessive release of glutamate that leads to excitotoxic damage to neurons (Faden et al., 1989; Katayama et al., 1990; Meldrum, 2000).

TBI is also associated with a range of deleterious consequences such as edema (Bouma and Muizelaar, 1992; Kochanek et al., 1997) and metabolic dysfunction (Verweij et al., 2000; Xiong et al., 1997) as well as second insults such as seizures (Asikainen et al., 1999; Vespa et al., 2010) and hypoxia (Davis et al., 2004; Davis et al., 2009; Manley et al., 2001; Miller et al., 1978; Schmoker et al., 1992). Second insults are common following a severe TBI with as many as one third of the patients arriving in the emergency department with significant hypoxia and hypotension (Manley et al., 2001). Second insults are frequently associated with poor outcome. For example, a combination of hypotension and elevated ICP results in an increased likelihood of a negative outcome including a persistently vegetative state or death (Marmarou et al., 1991). Furthermore, hypoxia (PaO₂ < 60 mmHg) or hypotension (SBP < 90 mmHg) are independently associated with increased morbidity and mortality following severe TBI (Chesnut et al., 1993). Second insults, such as hypoxemia and ischemia compound the accumulation of extracellular glutamate, sometimes increasing concentrations for hours following the primary insult (Bullock et al., 1998). Some of the complications specific to post-TBI hypoxia include increased neuronal damage (Bauman et al., 2000; Clark et al., 1997; Feng et al., 2012b; Nawashiro et al., 1995), exacerbated axonal pathology and neuro-inflammatory response (Goodman et al., 2011; Hellewell et al., 2010), and exacerbated sensorimotor and cognitive deficits (Bauman et al., 2000; Clark et al., 1997).

N-acetylaspartylglutamate (NAAG) is an abundant peptide neurotransmitter found in millimolar concentrations in the mammalian brain (Coyle, 1997; Neale et al., 2000), and, when released, selectively activates the group II metabotropic glutamate receptor subtype 3 (mGluR3) reducing the release of glutamate into the synapse (Sanabria et al., 2004; Xi et al., 2002; Zhao et al., 2001; Zhong et al., 2006). Once in the synapse, NAAG is rapidly hydrolyzed to NAA and glutamate by the NAAG peptidase catalytic enzymes, glutamate carboxypeptidase II and III (GCP II and GCP III) (Bzdega et al., 2004; Luthi-Carter et al., 1998). We have previously demonstrated that NAAG peptidase inhibitors reduce the accumulation of glutamate and improve neuronal and astrocytic survival when administered at the time of the TBI (Zhong et al., 2005; Zhong et al., 2006), or 30 minutes following (Feng et al., 2011) the injury. Furthermore, a NAAG peptidase inhibitor administered 30 minutes following TBI combined with hypoxic insult significantly reduced both acute neuronal and astrocytic cell death (Feng et al., 2012a). In the present study we tested the effects of the NAAG peptidase inhibitor PGI-02776 on motor and cognitive function as well as hippocampal neuronal survival in the weeks following fluid percussion TBI combined with a hypoxic second insult.

2. Results

2.1. Descriptive parameters

There were no significant differences between groups in pre-injury body weight or in temporalis or rectal temperature either pre- or post-injury (Table 1). Both vehicle and PGI-treated TBI rats received a similar magnitude of injury (Table 1) and a total of 4 animals died acutely following TBI with hypoxia (1 vehicle and 3 PGI-02776-treated). Over the first four days following injury both vehicle and PGI-02776-treated animals experienced weight loss. After the fourth day post-injury there was no difference in average daily weight gain between the sham, vehicle and PGI-02776-treated TBI groups. Sample sizes for behavior were $n = 6$, $n = 8$ and $n = 8$ for sham, TBI with hypoxia vehicle-treated, and TBI with hypoxia PGI-02776-treated respectively.

2.2. Motor Tasks

There was a significant effect of group on average duration spent on the rota-rod over the first two weeks following injury ($F_{(2,19)} = 4.47$, $p < 0.05$; Figure 1A). Both the vehicle-treated and PGI-02776-treated groups had similar reductions in performance (shorter durations) on day 1 post-TBI. Over days 1–15 post-TBI, vehicle-treated animals spent significantly less time on the rota-rod compared to sham controls ($p < 0.05$). In contrast, the performance of PGI-02776-treated rats over days 1–15 post-TBI was not statistically different from sham controls ($p = 0.51$). However, there was no statistical difference in performance between vehicle-treated and PGI-02776-treated rats.

Similar to the rota-rod, there was a significant effect of group on the latency to reach the goal box on the beam walk task over the first two weeks following injury ($F_{(2,19)} = 12.9$, $p < 0.001$; Figure 1B). Both the vehicle-treated and PGI-02776-treated groups had similar reductions in performance (increased latency) on day 1 post-TBI. In addition, both vehicle-treated ($p < 0.001$) and PGI-02776-treated ($p < 0.05$) rats had significantly increased mean latencies to reach the goal box as compared to sham controls over the two-week post-TBI period. There was a trend, however, for improved performance in the PGI-02776 group as compared to the vehicle-treated group ($p = 0.097$).

2.3. Morris Water Maze

There was a significant effect of group on latency to find the platform in the MWM ($F_{(2,19)} = 14.8$, $p < 0.001$; Figure 2). Post-hoc analysis revealed significant performance deficits (increased latency) in both the vehicle-treated ($p < 0.001$) and the PGI-02776-treated ($p < 0.05$) groups as compared to sham controls. However, treatment with PGI-02776 significantly improved performance as compared to vehicle ($p < 0.05$).

2.4. Hippocampal Cell Counts

There was a significant effect of group on estimated neuronal number in the CA2/3 of the hippocampus ($F_{(2,16)} = 6.3$, $p < 0.01$; Figure 3A). Post-hoc analysis detected a significant decrease in estimated neurons in the vehicle-treated group ($35,238 \pm 3840$) as compared to sham-injured group ($49,727 \pm 3183$; $p < 0.01$). While not significant, there was a trend towards a reduction in CA2/3 neurons in the PGI-02776-treated group ($39,511 \pm 1519$; $p = 0.07$). PGI-02776 did not increase the estimated number of neurons as compared to the vehicle treatment ($p = 0.92$). The average coefficient of error for CA2/3 estimations of neuronal number was 0.082 ± 0.002 with a range of 0.07 – 0.11.

There was also a significant effect of group on estimated neuronal number in the CA1 of the hippocampus ($F_{(2,16)} = 3.8$, $p < 0.05$; Figure 3B). However, post-hoc analysis revealed only a trend toward a reduction in estimated neuronal number in vehicle ($58,874 \pm 2986$; $p =$

0.10) and PGI-02776-treated groups ($58,784 \pm 3719$; $p = 0.08$) as compared to the sham-injured group ($75,116 \pm 6937$). PGI-02776 did not increase estimated cell number as compared to vehicle ($p = 0.99$). The average coefficient of error for the estimation of neuronal number was 0.055 ± 0.002 with a range of 0.04 – 0.07.

3. Discussion

In this study, rats were administered either saline or the urea-based NAAG peptidase inhibitor PGI-02776 (10 mg/kg, i.p.) 30 minute after moderate lateral fluid percussion TBI combined with 30 minutes of immediate hypoxia ($\text{FiO}_2 = 11\%$). We combined a 30 minute period of hypoxia immediately following TBI to model the clinical scenario of a paramedic arriving on the scene of an accident with the victim presenting with hypoxia and hypotension. Studies have found that over 50% of severe TBI patients suffered from inadequate oxygen saturation on the scene due in some cases to airway obstruction (Stocchetti et al., 1996) and that 25 to 33 percent of severe TBI patients arrive at the emergency department with significant hypoxia and hypotension (Manley et al., 2001). Similar to a previous study (Bramlett et al., 1999a), TBI with hypoxia produced significant sensorimotor deficits as well as cognitive behavioral deficits on the MWM. Post-TBI treatment with PGI-02776 significantly reduced long-term motor and cognitive deficits as compared to vehicle treated rats. While PGI-02776 did not prevent the very acute motor deficits caused by TBI with hypoxia, it did enhance recovery over the first two weeks following injury. These data highlight the potential of NAAG peptidase inhibitors to improve outcome following TBI with a hypoxic second insult.

One of the key sequelae following both TBI and TBI with a second insult is an increase in extracellular glutamate which can lead to excitotoxicity and subsequent cellular damage (Baker et al., 1993; Bullock et al., 1998; Chamoun et al., 2010; Meldrum, 2000; Vespa et al., 1998). One of the key targets of pre-clinical TBI research is to identify strategies to reduce extracellular glutamate accumulation, or the effects of elevated extracellular glutamate following injury in order to preserve neuroanatomy and function. A recently developed strategy targets the NAAG pathway in order to reduce the deleterious effects of glutamate excitotoxicity (Feng et al., 2011; Feng et al., 2012a; Neale et al., 2005; Orlando et al., 1997; Zhong et al., 2005; Zhong et al., 2006). NAAG is an abundant peptide in the central nervous system that is co-released with small amino acid transmitters, including glutamate and GABA, during intense neuronal stimulation (Coyle, 1997; Neale et al., 2000). When released, NAAG selectively binds and activates the presynaptic group II metabotropic glutamate receptor subtype 3 (mGluR3) (Neale et al., 2000; Schweitzer et al., 2000; Wroblewska et al., 1997; Wroblewska et al., 1998; Wroblewska, 2006), an autoreceptor, and reduces further release of neurotransmitter. Therefore, NAAG activates a negative feedback loop (Sanabria et al., 2004; Xi et al., 2002; Zhao et al., 2001; Zhong et al., 2006) that has the potential to reduce the accumulation of excess extracellular neurotransmitter following an injury without impairing physiological neuronal transmission. The NAAG peptidase catalytic enzymes GCP II and GCP III hydrolyze NAAG, breaking it down into its component products, NAA and glutamate (Bzdega et al., 2004; Luthi-Carter et al., 1998), thereby contributing to the pool of extracellular glutamate. NAAG hydrolysis also limits the availability of synaptically-released NAAG to activate the mGluR3 negative feedback loop which modulates (limits) presynaptic glutamate release. Several studies have demonstrated that inhibition of GCP II and GCP III increases the extracellular levels of NAAG, moderates the release of glutamate, and reduces excitotoxicity following ischemic insults in rodents (Neale et al., 2005; Tsukamoto et al., 2007) and TBI in rats (Zhong et al., 2005, 2006; Feng et al., 2011, 2012a).

Previously we have reported that following a moderate lateral fluid percussion TBI, administration of the NAAG peptidase inhibitor ZJ-43 increased the concentration of NAAG in the synapse and reduced the accumulation of glutamate, aspartate and GABA in the extracellular space as determined by microdialysis (Zhong et al., 2006). When the mGluR3 antagonist LY341495 was co-administered with ZJ-43, NAAG concentrations remained high while concentrations of glutamate, aspartate and GABA increased. These data confirmed that NAAG peptidase inhibitors were reducing neurotransmitter release specifically through increasing NAAG concentrations, activation of the mGluR3 receptor and ultimately presynaptic inhibition. Additional studies demonstrated that when administered three times at 0, 8 and 16 hr following TBI, ZJ-43 significantly reduced acute neuronal degeneration and acute loss of GFAP immunoreactivity (Zhong et al., 2005). Similar to the microdialysis study, co-administration of LY341495 significantly reduced the neuroprotective effects of ZJ-43.

PGI-02776, a di-ester prodrug modification of ZJ-43 (Olszewski et al., 2004; Yamamoto et al., 2004; Zhong et al., 2005; Zhong et al., 2006) was designed to enhance blood-brain barrier (BBB) penetration of the NAAG peptidase inhibitor. When administered to naïve mice, PGI-02776 (100 mg/kg) crosses the BBB with concentrations peaking at 2 hours and remains significantly elevated for 6 hours following injection (Feng et al., 2011). TBI is associated with compromised blood-brain barrier integrity which would enhance passage of blood-borne compounds into the brain parenchyma (Jiang et al., 1992). Similar to what was observed in studies using the parent ZJ-43 compound, PGI-02776 administered 30 minutes following moderate lateral fluid percussion TBI reduced acute cell death (Feng et al., 2011). Furthermore, PGI-02776-treated animals had improved cognitive performance in the Morris water maze (Feng et al., 2011). Subsequent studies demonstrated that treatment with PGI-02776 significantly reduced acute neuronal and astrocytic cell death 24 hours following a moderate lateral fluid percussion TBI combined with a 30-minute hypoxic second insult (Feng et al., 2012a). Therefore, our current data combined with our previous studies demonstrate that both the parent compound ZJ-43 and its prodrug PGI-02776 as NAAG peptidase inhibitors can reduce acute neuronal death (Feng et al., 2011; Feng et al., 2012a; Zhong et al., 2005) and improve cognitive function following either a moderate TBI (Feng et al., 2011) or a moderate TBI with a hypoxic second insult.

One interesting finding in both this and our previous studies is that NAAG peptidase inhibitors reduce acute neuronal and glial pathology (Feng et al., 2011; Feng et al., 2012a; Zhong et al., 2005) but in both a TBI alone (Feng et al., 2011) and our current TBI with hypoxia model, acute Fluoro-Jade reactivity did not translate to significant increases in CA2/3 or CA1 neuronal survival in the hippocampus 2 weeks following injury. One potential explanation is that NAAG peptidase inhibitors are delaying rather than reducing neuronal cell death. What is critical, however, is that, regardless of reduction in the estimated number of neurons, treatment with NAAG peptidase inhibitors improved motor and cognitive function in animals with TBI (Feng et al., 2011) or TBI with hypoxia in the present study. In fact, previous studies demonstrated that, following TBI, rodents can exhibit behavioral deficits in the absence of significant cell death (Gurkoff et al., 2006; Lyeth et al., 1990; Scheff et al., 1997) suggesting that dysfunctional neurons are sufficient to adversely influence behavioral outcome. It is possible, therefore, that NAAG peptidase inhibitors do not prevent the most severely injured cells from dying, but may have protected those neurons that might otherwise become dysfunctional and thus, lead to improved behavioral outcome.

TBI patients, and particularly patients with severe TBI, may have elevated extracellular levels of glutamate for hours following injury (Bullock et al., 1998; Chamoun et al., 2010). Furthermore, elevated mean extracellular glutamate levels in severe TBI patients are

associated with lower scores on the Glasgow outcome scale (Koura et al., 1998), and, when elevated for extended durations, increased mortality (Chamoun et al., 2010). Second insults may further increase glutamate concentrations both in magnitude and duration (Bullock et al., 1998) and second insults are frequently associated with poor outcome. An early clinical review identified that hypotensive second insults paired with increased ICP, significantly increased the likelihood of producing a vegetative state or death (Marmarou et al., 1991). In addition, hypoxia (PaO₂ < 60 mmHg) or hypotension (SBP < 90 mmHg) are independently associated with increased morbidity and mortality following severe TBI (Chesnut et al., 1993). Analysis of patients arriving in the emergency department with TBI found that as many as one third have had at least one significant hypoxic and hypotensive event (Manley et al., 2001). In animal models post-TBI hypoxia increases neuronal damage (Bauman et al., 2000; Bramlett et al., 1999b; Clark et al., 1997; Feng et al., 2012b; Nawashiro et al., 1995), prolongs metabolic dysfunction (Bauman et al., 2005), extends the duration of hypotension (Feng et al., 2012b), exacerbates axonal pathology and neuro-inflammatory responses (Goodman et al., 2011; Hellewell et al., 2010), and exacerbates sensorimotor and cognitive deficits (Bauman et al., 2000; Bramlett et al., 1999a; Clark et al., 1997). Furthermore, when TBI is accompanied by a second hypoxic insult, extracellular glutamate remains significantly elevated over time as compared to animals receiving TBI alone (Matsushita et al., 2000). These data highlight the negative impact of hypoxia on TBI patients and the necessity for additional pre-clinical studies designed to assess pharmacological interventions in TBI models with second insult.

Based on our understanding of the role of elevated extracellular glutamate and excitotoxicity in the hours following TBI, reducing glutamate and restoring homeostasis remains a prime target for pharmacological intervention. A major limitation of traditional anti-glutamatergic therapeutic targets including receptor blockade is that both physiological and pathophysiological activity is blocked. NAAG peptidase inhibitors were designed to increase the level of NAAG in the synapse, activation of the mGluR3, and ultimately reduce glutamate release. NAAG is only released into the synapse in response to relatively intense neuronal stimulation. Thus, one of the advantages of this strategy is that NAAG activation of mGluR3 only plays a substantial role when excessive glutamate is released and thus, does not disrupt physiological transmission of information via glutamate release. Furthermore, in cases where cells have significant calcium accumulation or physical damage, NAAG inhibitors will not prevent those cells from dying. In fact our data demonstrate that NAAG peptidase inhibitors reduce the acute pathology associated with moderate lateral fluid percussion with hypoxia, and improve long-term outcome even in the absence of a significant increase in estimated neuronal number in the hippocampus. These data, therefore, support the continued development of NAAG peptidase inhibitors as therapeutics to reduce glutamate-induced excitotoxicity following TBI and TBI with second insults.

4. Experimental Procedures

4.1. Subjects

Twenty-six male Sprague-Dawley rats (Harlan) weighing 320–350 g were used in this study. Animals were housed in individual cages in a temperature (22 °C) and humidity-controlled (50% relative) animal facility with a 12 h light/dark cycle. Animals had free access to food and water during the duration of the experiments. Animals remained in the animal facility for at least 7 days prior to surgery. The Institutional Animal Care and Use Committee at the University of California at Davis approved all animal procedures in the following experiments.

4.2. Experimental design

Animals were subjected to either a sham injury or moderate lateral fluid percussion TBI. Immediately after TBI, rats were ventilated under hypoxic conditions ($F_iO_2 = 11\%$) for 30 minutes (see section 4.5). Sham rats were ventilated under “experimental normoxia” conditions ($F_iO_2 = 33\%$) for 30 minutes. Immediately following the cessation of hypoxia, TBI rats were injected either with sterile saline (control) or 10 mg/kg PGI-02776. TBI rats were then compared to sham rats across motor and cognitive behavioral tests as well as stereologic estimations of neuronal number in the CA2/3 and CA1.

4.3. Lateral Fluid Percussion Surgery

As previously described (Feng et al., 2012b), rats were anesthetized with 4% isoflurane in a carrier gas mixture of nitrous oxide/oxygen (2:1 ratio), intubated, and mechanically ventilated ($F_iO_2 = 33\%$) with a rodent volume ventilator (Harvard Apparatus model 683, Holliston, MA). A surgical level of anesthesia was maintained with 2% isoflurane. Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, and a 4.8-mm diameter craniectomy was performed on the right parietal bone (centered at 4.5mm Bregma and lateral 3.0mm on the right side). A rigid plastic injury tube (modified Luer-loc needle hub, 2.6 mm inside diameter) was secured over the exposed, intact dura with cyanoacrylate adhesive. Two skull screws (2.1 mm diameter, 6.0 mm length) were placed into burr holes, 1 mm rostral to Bregma and 1 mm caudal to Lambda. An injury tube was secured to the skull and screws using cranioplastic cement (PlasticsOne, Roanoke, VA). Rectal temperature was continuously monitored during surgical preparation by a feedback temperature controller pad (CWE model TC-1000, Ardmore, PA) and was maintained within normal ranges. Temporalis muscle temperature was measured by insertion of a 29-gauge needle temperature probe (Physitemp unit TH-5, probe MT-29/2, Clifton, NJ) between the skull and temporalis muscle.

4.4. Lateral Fluid Percussion Injury

Experimental TBI was produced using a fluid percussion device (VCU Biomedical Engineering, Richmond, VA) (Dixon et al., 1987) using the lateral orientation (Feng et al., 2012b; McIntosh et al., 1989). The fluid percussion device consists of a Plexiglas cylindrical reservoir filled with isotonic saline. One end of the reservoir has a Plexiglas piston mounted on O-rings. The opposite end has a transducer housing with a 2.6 mm inside diameter male Luer-loc opening. Injury was induced by the impact of a pendulum on the piston, which injects a small volume of saline epidurally into the closed cranial cavity in the rat on the opposite end of the device, producing a brief displacement and deformation of neural tissue. The resulting pressure pulse was measured in atmospheres (ATM) by an extracranial transducer (model SPTmV0100PG5W02; Sensym ICT) and recorded on a digital storage oscilloscope (model TDS1002; Tektronix Inc., Beaverton, OR). Immediately prior to injury, rats were disconnected from the ventilator, the injury tube connected to the fluid percussion cylinder, and a moderate fluid percussion pulse (2.12–2.16ATM) was delivered within 10 sec. Immediately after injury, rats were returned to ventilation with 1% isoflurane in hypoxic carrier gas conditions (see section 4.5). The plastic injury tube and skull screws were removed during the hypoxic ventilation and the scalp incision was closed with 4-0 braided silk sutures.

4.5. Ventilation and controlled hypoxia

While normoxia in room air has an $FiO_2 = 21\%$, we elected to maintain sham rats on what we describe as “experimental normoxia”. During all surgical preparations rats were maintained with a mixture of nitrous oxide/oxygen (2:1) which produces an $FiO_2 = 33\%$ and has been used in many rat TBI studies (Feng et al., 2012a; Lyeth et al., 1993; Phillips et al.,

1994). Hypoxia was maintained in TBI rats with a carrier gas mixture of nitrous oxide/air (1:1) to produce an $F_iO_2 = 11\%$. After 30 min of ventilation following injury, isoflurane was discontinued and animals were extubated as soon as spontaneous breathing was observed.

4.6. Preparation and administration of PGI-02776

PGI-02776, a di-ester prodrug form of the urea-based NAAG peptidase inhibitor ZJ-43 (Feng et al., 2011), was synthesized and generously donated by the laboratory of Dr. Zhou (PsychoGenics, Tarrytown, NY). PGI-02776 (10 mg/mL) was dissolved in sterile 0.9% saline and injected intraperitoneal (i.p.) at a volume of 1 mL/kg. Either PGI-02776 or an equal volume of saline was administered 30-min following TBI coinciding with the cessation of hypoxia.

4.7. Motor Tasks

Rota-rod—Components of ambulatory motor coordination and exercise capacity were assessed using the rota-rod device (Ugo Basile, model 7700, Italy). Training consisted of four, 4-minute trials over the 2 days immediately preceding injury (2 trials/day). During each trial, animals were placed on a continuously rotating rod (59 mm diameter, 10 rotations/min) for 4 minutes. If an animal fell from the rod it was immediately placed back on. After 4 minutes animals were returned to their home cage. Immediately prior to surgery, baseline rota-rod performance was assessed. Each animal was given 3 trials, each trial separated by 5 minutes. For each trial animals were placed on a continuous rotating rod for up to 2 minutes. If an animal fell from the rod, the time was recorded and the animal was returned to its home cage. If, after 2 min, the animal was still on the rod, it was removed and returned to its home cage. The three trials were averaged to establish baseline. On post-injury days 1, 4, 7, 11 and 15 animals were tested on the rota-rod. The post-injury testing was similar to the baseline assessment and scores were determined by averaging performance over each test days set of three, 2-minute trials.

Beam Walk—Components of vestibulomotor function and fine motor coordination were assessed using a beam-walking task. The beam walk consisted of a 2.5 cm wide, 1.22 m long beam suspended 1 m above the floor. A foam pad covered the floor below the beam. Four steel pegs (3.0 mm diameter, 4.0 cm high) were evenly placed in an alternating sequence along the beam to increase the difficulty of the task. A baseline was established immediately prior to fluid percussion surgery. Performance was assessed by measuring the animal's latency to traverse the beam on days 1, 4, 7, 11 and 15 after TBI. Data for each daily session consisted of the mean of three trials.

Rats were trained on two consecutive days prior to TBI to escape a bright light and loud white noise by traversing the beam to enter a darkened goal box at the opposite end of the beam. In order to reduce anxiety, the aversive consequences of falling off of the beam were reduced by never allowing the animals to completely fall to the floor from the beam. Investigators stayed near by the animals to catch them if they began to fall. Animals were initially trained with the beam pegs removed and as they displayed proficiency, pins were added one at a time until each of the four pins were in place. Each animal received a series of trials until reaching a criterion of completing three consecutive trials in fewer than 10 sec each.

4.8. Spatial Learning

Acquisition of spatial learning and memory was assessed in the MWM on days 11–15 after TBI (Morris, 1984). The test apparatus consisted of a large white circular tank (220 cm diameter by 60 cm high) filled with water to a depth of 22 cm. Water temperature was maintained at 24–28 °C. A transparent circular escape platform (12.8 cm diameter, 20 cm

high) was placed in a fixed position in the tank 2 cm below the water surface. Four consistent visual cues were located in the test room outside of the maze. Rats were released from one of four starting points (selected randomly on each day for each rat) and allowed 120 s to find and mount the escape platform. If a rat did not find the platform within 120 s, the experimenter placed the rat on the platform. Rats remained on the platform for 30 s before being removed from the maze. Subjects received a 4-min inter-trial interval in a warmed holding cage before being returned to the maze for subsequent trials. Rats received a total of 4 trials per day, one from each starting point, over 5 consecutive days. Mean latency to find the platform was calculated for each day to assess learning. Data from all trials were recorded using a video tracking system (Poly-Track Video Tracking System version 2.1, San Diego Instruments Inc. San Diego, CA).

4.9. Tissue Collection and Sectioning

Rats were euthanized on post-injury day 16 with a diluted solution of SomnaSol® (150 mg/kg sodium pentobarbital; 25 mg/kg sodium phenytoin, Butler Schein, Dublin, OH) and then transcardially perfused with 100 mL of 0.1 M sodium phosphate buffer (pH = 7.4) followed immediately by 350 mL of 4% paraformaldehyde (pH 7.4). Brains were removed and post-fixed for 24 h in 4% paraformaldehyde at 4°C then immersed in 10% sucrose solution for 24 h followed by 48 h in a 30% sucrose solution. After buffer exchange, brains were rapidly frozen on powdered dry ice, and 45 µm coronal sections were cut at on a sliding microtome (American Optical, Model 860). Serial sections were saved in 24-well cell culture plates starting at -2.12 mm Bregma and ending at -4.80 mm Bregma. Every fifth section, starting from a random selection from the first 5 sections, throughout the hippocampus was then mounted on gelatin-coated glass microscope slides.

4.10. Cresyl Violet Staining

Sections were dehydrated at room temperature by immersion in a succession of ethanol baths: 70% (2 min × 1), 95% (2 min × 2) and 100% (2 min × 2) followed by xylene treatment for 16 min. Sections were then rehydrated in 100% (2 min × 2), 95% (2 min × 2) and 75% (2 min × 1) ethanol baths and rinsed with distilled water (30 sec × 2). Sections were stained with Cresyl violet acetate (0.1%) for 6 min, rinsed in distilled water (15 sec × 2), differentiated in 95% ethanol with 0.15% Acetic Acid, and dehydrated in 95% (30 sec × 2), and 100% (30 sec × 2) ethanol and xylene (5 min × 2). After dehydration, the sections were cover-slipped with Permount (Thermo-Fisher Scientific, Waltham, MA).

4.11. Stereological Cell Counts

Serial Cresyl Violet stained tissue sections were analyzed using stereology to estimate the total number of dorsal hippocampal CA2/3 and CA1 pyramidal neurons ipsilateral to the fluid percussion injury. Due to histological error, two vehicle-treated TBI rats and one PGI-02776-treated TBI rat were excluded from analysis resulting in an n = 6 brains each for sham and vehicle-treated rats and an n = 7 for PGI-02776-treated rats. Estimation of the total number of neurons in the ipsilateral hippocampus was made on a brightfield microscope (Nikon E600, Nikon, Tokyo) with a motorized stage (Bioprecision2, Ludl Electronic Products, Inc., Hawthorne, NY) using stereological software (Stereo Investigator™ 8.0, Microbrightfield, Inc., Williston, VT). For the CA2/3 counts, the region of interest was defined as the stratum pyramidale of the hippocampal CA2/3 with the borders of the region defined as the pyramidal layer entry into the dentate gyrus at the lateral tips of the dorsal and ventral blades of the dentate granule cells and the narrowing of the stratum pyramidale at the intersection of the CA1 to CA2. For the CA1 counts, the borders were defined as the intersection of the stratum pyramidale of the CA1 to CA2 through the most medial aspects of the CA1. The regions of interest were outlined using a 10X objective (Plan Apo, NA 0.45, Nikon). Neuronal cell counting was performed with a 100X oil immersion objective

(Plan Apo, NA 0.95, Nikon). The criterion for selection and quantification of surviving neurons was a morphologically distinct neuronal cell body. Both the number of CA2/3 and CA1 neurons were estimated on every 5th section resulting in an SSF = 0.2. CA2/3 neurons were counted in $25 \times 25 \mu\text{m}^2$ frames over a $150 \times 150 \mu\text{m}^2$ grid resulting in a ASF = 0.028. CA1 neurons were counted in $25 \times 25 \mu\text{m}^2$ frames over a $125 \times 125 \mu\text{m}^2$ grid resulting in a ASF = 0.04. For both the CA2/3 and CA1 the average section thickness was $\sim 15 \mu\text{m}$ and, after excluding guard zones $\sim 10 \mu\text{m}$ were counted resulting in a TSF = 0.67.

4.12. Statistical Analysis

Data analysis was performed using SPSS software (Version 20, Chicago IL), which adheres to a general linear model. Alpha level for Type I error was set at 0.05 for rejecting null hypotheses. All data are expressed as mean $\pm$ standard error of the mean (SEM). Performance on the rota-rod, beam walk and MWM were analyzed with repeated measures ANOVA, with average daily values used as the repeated measure within groups variable. A Bonferroni post-hoc analysis was used to compare differences among the three groups. The stereological estimate of the number of neurons between groups was analyzed with a one-way ANOVA with a Bonferroni post-hoc analysis used to compare differences among the three groups.

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reduces glutamate, aspartate and GABA levels in the dorsal hippocampus following fluid percussion injury in the rat. *J Neurochem.* 2006; 97:1015–25. [PubMed: 16606367]

Research Highlights

- TBI plus hypoxia produced significant motor and cognitive deficits in rats.
- Post-injury administration of PGI-02776 reduced motor deficits after TBI.
- Post-injury administration of PGI-02776 reduced cognitive deficits after TBI.

Figure 1.

Motor Behavior. **(A)** Rota-rod: The vehicle treated TBI group had a significantly shorter average duration of time spent on the rota-rod as compared to the sham injured group. **(B)** Beam Walk: Both the vehicle and PGI-02776-treated TBI groups had significantly longer average latency to reach the escape box. There was a trend toward an improvement in behavior in the PGI-treated TBI group as compared to the vehicle treated TBI group on the beamwalk ($p < 0.097$). * $p < 0.05$ as compared to the sham-injured group.

Figure 2.

Morris Water Maze. Both vehicle and PGI-02776-treated TBI groups had significantly longer latencies to find the escape platform in the MWM as compared to the sham-injured group. PGI-02776-treated TBI rats found the platform significantly faster than the vehicle-treated TBI group. * $p < 0.05$ as compared to sham injured group. † $p < 0.05$ as compared to the PGI-02776 treated groups

Figure 3.

Hippocampal Stereology. **(A) CA2/3:** There were significantly fewer estimated CA2/3 neurons in vehicle-treated TBI as compared to the sham-injured group. There was a trend toward significantly fewer surviving CA2/3 neurons in the PGI-02776-treated TBI group ($p = 0.07$). **(B) CA1:** There was a trend toward a reduction in the estimated number of CA1 neurons in both the vehicle-treated ($p = 0.1$) and PGI-02776-treated ($p = 0.08$) TBI groups as compared to the sham-injured group. * $p < 0.05$ as compared to the sham injured group

Summary of descriptive data. ATM = injury magnitude in atmospheres, pre = 1 min prior to TBI, post = 1 min following TBI.

Table 1

	Weight	ATM	Temporalis Temp.		Rectal Temp.	
			pre	post	pre	post
Sham	313.0±18.8		35.5±0.1	35.4±0.1	36.8±0.2	36.8±0.2
Vehicle	295.7±12.0	2.14±0.01	35.7±0.3	35.7±0.3	37.2±0.5	37.3±0.3
PGI-02776	288.7±10.8	2.13±0.02	35.4±0.3	35.7±0.3	36.9±0.3	37.1±0.2