17 β -Estradiol Confers Protection after Traumatic Brain Injury in the Rat and Involves Activation of G Protein-Coupled Estrogen Receptor 1

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Abstract

Traumatic brain injury (TBI) is a significant public health problem in the United States. Despite preclinical success of various drugs, to date all clinical trials investigating potential therapeutics have failed. Recently, sex steroid hormones have sparked interest as possible neuroprotective agents after traumatic injury. One of these is 17 β -estradiol (E2), the most abundant and potent endogenous vertebrate estrogen. The goal of our study was to investigate the acute potential protective effects of E2 or the specific G protein-coupled estrogen receptor 1 (GPER) agonist G-1 when administered in an intravenous bolus dose 1 hour post-injury in the lateral fluid percussion (LFP) rodent model of TBI. The results of this study show that, when assessed at 24 hours post-injury, E2 or G-1 confers protection in adult male rats subjected to LFP brain injury. Specifically, we found that an acute bolus dose of E2 or G-1 administered intravenously 1 hour post-TBI significantly increases neuronal survival in the ipsilateral CA 2/3 region of the hippocampus and decreases neuronal degeneration and apoptotic cell death in both the ipsilateral cortex and CA 2/3 region of the hippocampus. We also report a significant reduction in astrogliosis in the ipsilateral cortex, hilus, and CA 2/3 region of the hippocampus. Finally, these effects were observed to be chiefly dose-dependent for E2, with the 5 mg/kg dose generating a more robust level of protection. Our findings further elucidate estrogenic compounds as a clinically relevant pharmacotherapeutic strategy for treatment of secondary injury following TBI, and intriguingly, reveal a novel potential therapeutic target in GPER.

Key words: apoptosis; estrogen; lateral fluid percussion; neuronal degeneration; neuroprotection

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a significant public health problem in the United States. Annually, approximately 1.7 million TBIs are incurred, 53,000 people die, and 3.2–5.3 million others are living with long-term disabilities as a result. Despite preclinical successes, to date all clinical trials investigating potential therapeutics have failed.¹ In addition, the financial burden associated with TBI is estimated at roughly \$60 billion a year.^{2,3} TBI-induced biomechanical (primary injury) and neurochemically-mediated damage (secondary injury) often lead to deficits in cognitive, neuropsychiatric, and physical functioning.^{4–12} Secondary injury mechanisms remain targets in the pathophysiology of TBI that could be manipulated by therapeutic interventions for prevention of further cell destruction and dysfunction. Thus, there is a significant unmet need for novel drug therapies that efficaciously target aspects of secondary injury.

Recently, sex steroid hormones have sparked interest as possible therapeutic agents following traumatic injury. One of these is 17 β -

estradiol (E2), the most abundant and potent endogenous vertebrate estrogen. Our research group has previously reported that E2 administration confers protection in models of spinal cord injury (SCI) and severe blood loss.^{13–21} In prior TBI research, E2 has been shown to reduce cortical contusion volumes, apoptosis, blood-brain barrier permeability, edema, levels of pro-inflammatory cytokines, and intracranial pressure (ICP), as well as to upregulate expression of anti-apoptotic protein Bcl-2, increase cerebral perfusion pressure (CPP), and improve neurological scores.^{22–27} Taken together, these data suggest that E2 is protective and warrants further study as a potential therapeutic for treatment of TBI.

E2 signals through the classical estrogen receptors α and β (ER α ; ER β) and the recently characterized G-protein coupled estrogen receptor 1 (GPER), which binds E2 and various estrogenic compounds, including the GPER-specific agonist, G-1, and initiates rapid intracellular signaling events.^{28–30} However, GPER's role in the CNS has yet to be fully characterized, and its potential contribution to protection in TBI remains uninvestigated. Because GPER binds E2 as well as other more specific ligands, it could serve as a novel therapeutic target.

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The goal of our study was to investigate the acute potential protective effects of E2 or G-1 when administered in an intravenous bolus dose 1 hour post-injury in the clinically relevant lateral fluid percussion (LFP) rodent model of TBI. We found that in ipsilateral cortex and hippocampus, administration of E2 or G-1 post-TBI significantly increased cell survival and decreased neuronal degeneration, apoptosis, and reactive astrogliosis when assessed at 24 hours following TBI.

Methods

Animals

Adult male Sprague-Dawley rats (2 months old, 300–350 g; Charles River Laboratories International, Inc.) were housed two/cage on a 12-hour light/dark cycle in a temperature- (22°C) and humidity-controlled facility and allowed standard rat chow and water *ad libitum*. All animal care and experimental procedures complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. For generation of histology tissue, animals were divided into five groups. Uninjured control animals received craniectomy only. These subjects were treated with either vehicle ($n=4$) or E2 ($n=4$). As there were no significant differences observed between control groups treated with vehicle or E2, they were pooled for all analyses (SHAM, $n=8$). The injured groups were 1) vehicle-treated TBI (TBI VEH, $n=8$); 2) 1 mg/kg E2-treated TBI (TBI E2 1 mg, $n=8$); 3) 5 mg/kg E2-treated TBI (TBI E2 5 mg, $n=8$); and 4) 0.49 mg/kg G-1-treated TBI (TBI G1, $n=8$). For generation of Western blot tissue, an additional cohort of animals was examined: 1) uninjured sham control (SHAM, $n=4$); 2) vehicle-treated TBI (TBI VEH, $n=4$); 3) 5 mg/kg E2-treated TBI (TBI E2 5 mg, $n=4$); and 4) 0.49 mg/kg G-1-treated TBI (TBI G1, $n=4$). Animals were humanely euthanized 24 h post-TBI and tissue was extracted for histological evaluation or protein quantification.

Surgical procedure

Animals were anesthetized with 4% isoflurane gas in an O₂ carrier for 4 min, followed by an intraperitoneal injection of 100/10 mg/kg mixture of ketamine/xylazine; then anesthesia was maintained via ventilation with 1.5% isoflurane gas for the duration of surgery. Normothermia was maintained throughout surgery by keeping the animal on a water-jacketed heating pad. After securing the animal in a stereotaxic frame, a midline scalp incision was made and the skin and fascia reflected to expose the bregma, lambda, and sagittal sutures, as well as the lateral ridges. A 4.8-mm craniectomy was trephined over the right parietal cortex, midway between bregma and lambda, tangential to the sagittal suture, as previously described.³¹ A rigid plastic injury tube (modified female Luer-lock 20G needle hub) was bonded to the skull with cyanoacrylate adhesive over the open craniectomy with the dura intact and a stabilizing screw was placed in a burr hole drilled rostral to bregma on the ipsilateral side. The injury tube and stabilizing screw were secured with dental acrylic. Then the scalp was sutured, and the animal was returned to a warmed recovery cage.

Induction of lateral fluid percussion traumatic brain injury

Experimental TBI was induced the day following craniectomy, using a fluid percussion device (VCU Biomedical Engineering, Richmond, VA) as previously described.^{32–34} The device consists of a Plexiglas cylinder (60 cm in length and 4.5 cm in diameter) filled with sterile water. A piston is mounted on O-rings at one end and an extracranial pressure transducer (Entran Devices, Inc., EPN-0300A, Fairfield, NJ) connected to a storage oscilloscope (Tektronix, TDS 310, Beaverton, OR) is attached to the opposite

end. A 5-mm tube (internal diameter 2.6 mm) ending in a male Luer-loc is fitted at the end. The animal was anesthetized with 4% isoflurane gas for 4 min, and moderate TBI was induced by rapidly injecting a small volume of sterile saline into the closed cranial cavity over the right ipsilateral hemisphere with the fluid percussion device. Immediately after the impact, the animal was removed from the device, monitored for duration of apnea and unconsciousness, and re-sutured while receiving supplemental oxygen ventilation. The magnitude of the pressure pulse was measured by a pressure transducer, stored on an oscilloscope, and later converted to atmospheres (ATM). The pressure pulse was monitored and controlled in order to deliver an equivalent impact to each animal.

Administration of drug

At 30 min post-TBI, animals were anesthetized with 4% isoflurane gas for 4 min and then maintained on 2% isoflurane ventilation for the duration of drug delivery. An incision was made in the right inner flank and the femoral vein was isolated and cannulated using polyethylene tubing (PE50). At 1 h post-TBI, either E2 (1 mg/kg or 5 mg/kg β -estradiol 3,17-disulfate dipotassium salt in a carrier of sterile water, Sigma-Aldrich Co., St. Louis, MO) vehicle (sterile saline), or 0.49 mg/kg G-1 (Sigma-Aldrich) in a carrier of 10% EtOH and 90% sterile water was administered via the femoral vein. The 1 mg/kg dose of E2 had been previously reported to result in peak plasma estrogen levels of 500 ng/mL.³⁵ The G-1 dose of 0.49 mg/kg is the maximal dose soluble in a physiological vehicle. The femoral vein was then ligated with braided silk thread and the incision closed. The animal was returned to a warmed recovery cage.

Histological analysis

Tissue preparation. At 24 h post-TBI, animals were humanely euthanized with Fatal Plus (100 mg/kg i.p.; Vortech Pharmaceuticals, Dearborn, MI) and perfused intracardially with ice-cold 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde (PFA) for 20 min. Brains were harvested and post-fixed for 24 h at 4°C in 4% PFA, then subsequently cryoprotected in an increasing gradient of 10%–30% sucrose for 24 h at 4°C. The brains were marked with tissue dye over the right hemisphere, blocked and trimmed at 5 mm rostrally and 8 mm caudally, then embedded in OCTTM Compound (Tissue-Tek; Fisher Scientific, Pittsburgh, PA) and frozen in ice-cold 2-methylbutane. Tissue was stored at –80°C until serial random sectioning. Serial 50 μ m slices were sectioned on a cryostat (Leica Instruments, Nusloch, Germany) and collected from bregma –0.8 mm to –4.8 mm, encompassing the cortical region at the injury epicenter as well as the entire hippocampal formation.³⁶ The sections were mounted on 1% gelatin-coated slides and stored at –20°C until further histological analysis.

Cresyl violet histochemistry. Cresyl violet histological processing of tissue demarks Nissl substance, which is composed mostly of rough endoplasmic reticulum and is lost after neuronal injury or axonal degeneration.³⁷ For cresyl violet histochemistry, tissue was rinsed and dried overnight. Sections were dehydrated through graded alcohol to xylene for two changes of 5 min each, and then rehydrated through graded alcohol to water. Sections were then submerged in 0.1% aqueous cresyl fast violet (EM Science, Gibbstown, NJ) in a sodium acetate buffer for 4 min, followed by differentiation in 95% ethanol with 0.2% HCl for 5 min. Differentiation was timed such that both Nissl substance and cell nuclei were clearly visible. Slides were washed in graded alcohol and xylene and coverslipped with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

Fluoro-Jade B immunohistochemistry. Fluoro-Jade B is an anionic fluorescein derivative that binds to degenerating neurons.³⁸

Briefly, sections were rehydrated through graded ethanol to distilled water, then incubated in 0.06% potassium permanganate for 15 min to reduce nonspecific fluorescence. Tissue was rinsed in distilled water and processed with 0.006% Fluoro-Jade B in 0.1% acetic acid for 30 min at room temperature, then sections were washed with distilled water (1 min $\times$ 3) and dried for 30 min at 37°C, followed by drying at room temperature overnight. Finally, sections were rinsed in xylene (5 min $\times$ 2) and coverslipped with DPX mounting media (Electron Microscopy Sciences Inc., Hatfield, PA).

Caspase-3 immunohistochemistry. Caspase 3 acts as an effector caspase following activation via autoproteolytic cleavage or cleavage by other proteases as part of the programmed cell death cascade and is an indicator of extrinsic or intrinsic apoptosis. Briefly, slide-adhered sections were first washed in 0.1 M phosphate buffer (PB), then in 0.1 M PBS (10 min $\times$ 2). Nonspecific immunoreactivity was blocked with a solution of 3% normal goat serum, 0.3% Triton X, 3% bovine serum albumin (BSA), and 0.1 M PBS, and tissue was incubated for 40 min at 37°C, followed by 20 min at room temperature. Sections were rinsed in 0.1 M PBS, then incubated in anti-active caspase-3 primary antibody (Ab) diluent, which consisted of 1% normal goat serum, 0.3% Triton X, 2% BSA, anti-active caspase 3 Ab at a 1:200 titer (anti-active caspase 3; Abcam, Cambridge, MA) and 0.1 M PBS, for 48 h at 4°C. After primary Ab incubation, tissue was rinsed in 0.1 M PBS (15 min $\times$ 6), then placed in secondary Ab diluent containing 1% normal goat serum, 3% Triton X, 2% BSA, Alexa 568 secondary Ab at a 1:500 titer (goat anti-rabbit IgG; Invitrogen, Carlsbad, CA) and incubated for 24 h at 4°C. Following incubation with secondary Ab, sections were washed in 0.1 M PBS (15 min $\times$ 3), and slides were coverslipped with DPX mountant (Electron Microscopy Sciences Inc.).

Glial fibrillary acidic protein (GFAP) immunohistochemistry. Reactive glial response was determined by measuring the luminance intensity of GFAP immunoreactivity. Slide-adhered sections were washed in 0.1 M PB (10 min $\times$ 3) and then blocked in an endogenous peroxidase treatment (0.5% hydrogen peroxide in 0.1 M PB) for 30 min. Following washes in 0.1 M PB and PBS (5 min $\times$ 3), nonspecific background was blocked with a solution of 3% normal goat serum, 3% BSA, 0.3% Triton X, and 0.1 M PBS. Tissue was rinsed in 0.1 M PBS and incubated in a diluent mixture (1% normal goat serum + 2% BSA + 0.3% Triton X + 0.1 M PBS) containing anti-GFAP (Dako, Carpinteria, CA) at a 1:400,000 titer for 30 min at 37°C, then overnight at 4°C. Next, tissue was washed in 0.1 M PBS (10 min $\times$ 9), then incubated for 24 h at 4°C in the diluent mixture (described above) containing secondary Ab serum at a 1:400 titer (goat anti-rabbit Alexa Fluor 488; Invitrogen, Grand Island, NY). Sections were rinsed in 0.1 M PB (10 min $\times$ 3) and 0.1 M PBS (10 min $\times$ 6), then slides were coverslipped with DPX mountant (Electron Microscopy Sciences Inc.).

Unbiased stereology and quantification of histological markers. Beginning at a randomly chosen first section near bregma -0.8 mm, measurements were obtained in every tenth section throughout the rostral-caudal extent of the lesion, ending approximately at bregma -4.8 mm (~ 4 mm total tissue). All assessments were performed by investigators naïve to the treatment of the animal. Stereological counting was conducted on an Olympus BX-51 microscope linked to a MicroFire® true color CCD digital camera (Optronics, Goleta, CA) using StereoInvestigator software (Microbrightfield Inc., Williston, VT) at 200X–400X magnification. In the regions of interest, the optical fractionator probe was used to quantify the total number of neurons. For analysis of cresyl violet histochemistry, only neurons possessing a soma diameter greater than 9 μ m and a clearly defined nucleus and/or

nucleolus were counted. For assessment of Fluoro-Jade B and caspase 3 immunohistochemistry, only cells with fluorescence intensity twice that of background were counted. GFAP-positive cells were quantified using relative luminance intensity. This was calculated from the fluorescence intensity in three 50 $\times$ 50 μ m sampling boxes that were randomly placed in the regions of interest. Micrographs were taken with a 20X objective. All intensity values presented in the figures are raw data obtained from captured images. These pixel intensities were kept within the camera's dynamic range (0–4095) and pixel saturation was avoided by manipulating the imaging parameters of gain, offset, and exposure time to ensure that intensity values fell within the middle of the dynamic range. All images were captured with identical settings. Three intensity values per region were averaged.

Western blot analysis

Brains were extracted and hippocampi rapidly dissected out, collected, and immediately flash frozen on dry ice. Tissue was kept at -80°C until generation of tissue lysate. Hippocampi were mechanically homogenized in ice cold lysis buffer (100 mM Tris, pH 7.5, 1% sodium dodecyl sulfate (SDS)) containing protease inhibitors (Complete Mini Protease Cocktail Tablets, Roche Diagnostics, Indianapolis, IN) and then sonicated for 10 sec, followed by centrifugation at 14,000 g for 5 min at 4°C to pellet debris. Protein quantification of the supernatant was performed with the Bio-Rad DC protein assay kit (Hercules, CA) and protein diluted to a final concentration of 2 μ g/ μ L. An equal volume of 2x Laemmli sample buffer (Sigma-Aldrich Co.) was added to the protein and samples were placed in boiling water for 5 min. Protein was then loaded into a 10% gradient pre-cast SDS gel (Bio-Rad Mini-PROTEAN® TGX precast gel, Hercules, CA) and run at 100 V for 2 h, then transferred at 100 V for 1 h. Membranes were blocked overnight at 4°C in blocking buffer (5% milk in TBS-T), then incubated in primary Ab (anti-active caspase 3, 1:500; anti-GPR30/GPER, 1:250; anti- α -tubulin loading control, 1:10,000; Abcam, Cambridge, MA) at room temperature for 1 h. Following 3 $\times$ 10-min rinses, membranes were incubated in horseradish peroxidase-conjugated secondary Ab for 1 h at room temperature (goat anti-rabbit IgG, 1:2000, Bio-Rad; goat anti-mouse IgG, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), then washed again (3 $\times$ 10 min), developed with enhanced chemiluminescence (SuperSignal® West Femto Maximum Sensitivity Substrate kit, Thermo Scientific, Pittsburgh, PA), and imaged with the Kodak Image Station 4000 MM. Protein was quantified with UN-SCAN-IT gel™ Version 6.1 software (Silk Scientific Inc., Orem, UT) and total active caspase 3, and GPER protein were normalized to α -tubulin expression in the same lane. Relative protein expression is reported in arbitrary units.

Statistical analysis

All data were analyzed with SigmaPlot® (v11; Systat Software Inc., San Jose, CA) and are presented as mean $\pm$ SEM. One-way analysis of variance (ANOVA) tests were performed followed by Holm-Sidak *post-hoc* analysis for all pairwise multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Injury severity

The magnitude of the impact force produced by the fluid pressure pulse was recorded for each subject immediately following induction of injury. The impact force was compared between all treatment groups with a significant main effect of group determined ($F(4,39) = 1212.91$, $p < 0.001$). Pairwise comparisons showed that there was not a significant difference in delivered impact force

between the injury groups (TBI VEH, 5.61 ± 0.08 ; TBI E2 1MG, 5.61 ± 0.10 ; TBI E2 5MG, 5.61 ± 0.07 ; TBI G1, 5.63 ± 0.06) but that the uninjured SHAM control group was statistically different. The duration of transient unconsciousness following lateral fluid percussion TBI is an indicator of injury severity.^{33,34,39} Transient unconsciousness following induction of TBI was determined by monitoring the duration of suppression of the righting reflex. A main effect of group on righting time was found ($F(4,39) = 15.32$, $p < 0.001$). Pairwise comparisons demonstrated that animals in the uninjured SHAM control group exhibited significantly shorter durations of unconsciousness than each injury group (SHAM, 127.88 ± 1.55) and there were no significant differences in righting times between all groups that were subjected to TBI (TBI VEH, 634.38 ± 60.93 ; TBI E2 1MG, 641.13 ± 80.65 ; TBI E2 5MG, 631.38 ± 66.27 ; TBI G1, 641.93 ± 48.22). Taken together, these data indicate that all groups subjected to TBI received an injury of equivalent severity.

E2 or G-1 treatment increases neuronal survival in the hippocampus

In order to determine the effect of E2 or G-1 administered acutely post-injury on neuronal survival in the CA 2/3 region of the

hippocampus, we performed cresyl violet histochemistry on brain tissue extracted 24 h following induction of TBI and assessed the number of surviving neurons via unbiased stereology (Fig. 1). Uninjured SHAM controls exhibited normal anatomical structure of the entire hippocampal formation and individual cells in the CA 2/3 region of the hippocampus appeared morphologically intact with a well-defined cell soma, nucleus, and nucleolus (Fig. 1A and 1F). Tissue from animals that sustained TBI showed a reduction in viable neurons, presence of hemorrhage, and damage to the normal anatomical structure of the hippocampus on the side ipsilateral to injury (Fig. 1B–E, G–J). In the injury groups that received E2 or G-1, there appeared to be an increase in morphologically intact, surviving neurons in the CA 2/3 region of the hippocampus compared to the animals that only received vehicle. Stereological quantification followed by statistical analysis confirmed this observation with a main effect of group ($F(4,39) = 54.22$, $p < 0.001$). Pairwise comparisons revealed that there were significantly greater numbers of surviving neurons in the CA 2/3 region of the ipsilateral hippocampus in the injury group treated with the 5 mg/kg dose of E2 and the group that received G-1 compared to the injury group that received VEH (Fig. 1K). Furthermore, the number of surviving neurons quantified in the E2 5 mg/kg dose or G-1 group was not significantly different from that counted in the uninjured

FIG. 1. Effect of E2 or G-1 administration on neuronal survival in the CA 2/3 region of the ipsilateral hippocampus. Surviving neurons were quantified with unbiased stereology and representative micrographs are shown at 40X (entire hippocampal formation, (A–E); scale bar = 100 μ m) and 400X magnification (CA 2/3 region of the hippocampus only, (F–J); scale bar = 200 μ m). A and F represent the SHAM group, B and G the TBI VEH group, C and H the TBI E2 1 mg group, D and I the TBI E2 5 mg group, and E and J the TBI G1 group. The black box in A denotes the approximate region of interest presented as magnifications in F–J. Micrographs and cell counts were made from sections of tissue extracted at 24 h post-TBI. Quantification (mean scores $\pm$ standard error of the mean) of surviving neurons is presented in K (*significant difference compared to SHAM; +significant difference compared to VEH-treated injury group; **significant difference compared to E2 1 mg-treated injury group, $p < 0.001$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol; G1, G-1).

SHAM control group. These data indicate that E2 or G-1, when administered acutely following TBI, improves neuronal survival as measured at 24 h post-injury.

E2 or G-1 treatment reduces neuronal degeneration in cortex and hippocampus

To evaluate the effect of post-TBI E2 or G-1 administration on degenerating neurons, Fluoro-Jade B immunohistochemistry was conducted on brain tissue sections from the ipsilateral cortex and ipsilateral CA 2/3 region of the hippocampus (Fig. 2). We observed an increase in Fluoro-Jade B-positive staining in animals that were subjected to TBI and treated with vehicle compared to uninjured SHAM controls (Fig. 2B, G). This increase was attenuated in the injury groups that received E2 or G-1 (Fig. 2C–E, H–J; indicated by white arrows in G–J). Quantification via stereology demonstrated a main effect of group in the ipsilateral cortex ($F(4,39)=27.18$, $p<0.001$) and ipsilateral CA 2/3 ($F(4,39)=21.21$, $p<0.001$). Pairwise comparisons revealed that in the ipsilateral cortex the

numbers of degenerating neurons were significantly reduced in injury groups that received E2 and further reduced in animals administered G-1 compared to the vehicle-treated injured group (Fig. 2K). Similarly, in the ipsilateral CA 2/3, there was a significant decrease in degenerating neurons in injured animals that received E2 or G-1 compared to those that received vehicle, and this reduction was dose-dependent for E2 (Fig. 2L). These results indicate that an acute post-injury administration of E2 or G-1 reduces neuronal degeneration in the ipsilateral cortex and hippocampus.

E2 or G-1 treatment decreases apoptosis in cortex and hippocampus

To assess the effect of E2 or G-1 on apoptosis in the ipsilateral cortex and hippocampus following TBI, immunohistochemistry for active caspase 3 was performed and numbers of caspase 3-immunoreactive (Casp3-IR) cells were quantified with unbiased stereology (Fig. 3). In all injury groups, an increase in Casp3-IR cells was observed in both the ipsilateral cortex and CA 2/3 as

FIG. 2. Effect of E2 or G-1 administration on neuronal degeneration in the ipsilateral cortex and CA 2/3 region of the hippocampus. Representative micrographs of degenerating neurons are shown at 200X magnification (cortex, (A–E); CA 2/3 region of the hippocampus, (F–J); scale bar = 100 μ m) and numbers of dying cells were quantified with unbiased stereology (K–L). A and F represent the SHAM group, B and G the TBI VEH group, C and H the TBI E2 1 mg group, D and I the TBI E2 5 mg group, and E and J the TBI G1 group. White arrows indicate areas of degenerating neurons (G) attenuated in H, I, and J. Quantification (mean scores $\pm$ standard error of the mean) of degenerating neurons in cortex and in the CA 2/3 region of the hippocampus is presented in K and L, respectively (*significant difference compared to SHAM; +significant difference compared to VEH-treated injury group; **significant difference compared to E2 1 mg-treated injury group; #significant difference compared to E2 5 mg-treated injury group, $p<0.001$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol; G1, G-1).

FIG. 3. Effect of E2 or G-1 administration on programmed cell death in the ipsilateral cortex and CA 2/3 region of the hippocampus. Representative micrographs of caspase 3-immunoreactive (Casp3-IR) cells are shown at 200X magnification (cortex, (A–E); CA 2/3 region of the hippocampus, (F–J); scale bar = 100 μ m) and numbers of apoptotic cells were quantified with unbiased stereology (K–L). A and F represent the SHAM group, B and G the TBI VEH group, C and H the TBI E2 1 mg group, D and I the TBI E2 5 mg group, and E and J the TBI G1 group. White arrows denote areas in the CA 2/3 region of the ipsilateral hippocampus with increased numbers of Casp3-IR cells in G which are reduced in H and I (arrows omitted in J due to lack of observable immunoreactivity). Quantification (mean scores $\pm$ standard error of the mean) of Casp3-IR cells in cortex and in the CA 2/3 region of the hippocampus is presented in K and L, respectively (*significant difference compared to SHAM; + significant difference compared to VEH-treated group; **significant difference compared to E2 1 mg-treated group; $p < 0.001$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol, G1, G-1). Color image is available online at www.liebertpub.com/neu

compared to uninjured sham control animals (Fig. 3B, G). Injured animals that received E2 or G-1 exhibited a reduction of Casp3-IR cells compared to animals that were treated with vehicle (Fig. 3C–E, H–J; indicated by white arrows in G–I). Stereological quantification followed by statistical evaluation showed that there was a main effect of group for the ipsilateral cortex ($F(4,39) = 26.13$, $p < 0.001$). Pairwise comparisons indicated a significant reduction in Casp3-IR cells in groups administered E2 or G-1; additionally, subjects given G-1 exhibited significantly lower Casp3-IR cell counts than those receiving 1mg/kg E2 (Fig. 3K). In the ipsilateral CA 2/3 region of the hippocampus a main effect of group was also observed ($F(4,39) = 14.17$, $p < 0.001$) and pairwise comparisons showed that animals treated with E2 or G-1 exhibited a significant decrease in numbers of Casp3-IR cells compared to animals that received vehicle post-TBI, and these numbers were not significantly different from the uninjured SHAM controls (Fig. 3L). Taken together, these data indicate that acute post-injury administration of E2 or G-1 provides protection following TBI as it reduces apoptosis in both cortex and hippocampus.

E2 or G-1 administration reduces expression of active caspase 3 protein in the ipsilateral hippocampus

To augment our immunohistochemical analysis, and to evaluate the effect of E2 or G-1 treatment on changes in expression levels of active caspase 3 protein in the ipsilateral hippocampus, immunoblotting was employed and quantification of the relative amount of active caspase 3 protein was conducted (Fig. 4). Representative bands of active caspase 3 were observed at ~ 17 kDa in all treatment groups and are presented in Figure 4A. As a loading control, α -tubulin was used. We observed a significant effect of group ($F(3,15) = 6.52$, $p < 0.007$). Pairwise comparisons revealed increases in active caspase 3 protein in the injury group that received vehicle compared to the SHAM vehicle-treated animals (Fig. 4B). This injury-induced increase was reduced in both the E2 and G-1-treated groups; additionally, quantification of protein expression levels revealed that the values measured in these groups were not significantly different from those of SHAM. These data are similar to those generated

FIG. 4. Effect of E2 or G-1 administration on active caspase 3 protein expression levels in the ipsilateral hippocampus. Representative bands from Western blots are shown in (A). Quantification of total protein expression relative to α -tubulin in each lane is presented in (B), (*significant difference compared to SHAM; +significant difference compared to VEH-treated injury group; $p < 0.007$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol; G1, G-1; a.u., arbitrary units).

with the immunoreactivity analysis in Figure 3 and support the hypothesis that post-injury administration of E2 or G-1 reduces apoptosis.

E2 or G-1 treatment reduces reactive astrogliosis in cortex and hippocampal formation

To analyze the effect of E2 or G-1 administration post-TBI on reactive astrogliosis, GFAP immunohistochemistry was conducted and the relative luminance intensity of GFAP-immunoreactive (GFAP-IR) astrocytes was assessed in the ipsilateral cortex, CA 2/3 region of the hippocampus, and the hilus of the dentate gyrus (Fig. 5). Compared to the uninjured SHAM control group, animals in all injury groups appeared to express a robust increase in GFAP-IR astrocytes (Fig. 5B–E, G–J, and L–O). Injured animals that received the bolus dose of E2 in either the 1 mg/kg or 5 mg/kg dose or G-1 appeared to show a reduction in GFAP-IR astrocytes (representative astrocytes are indicated by white arrows in Fig. 5B–E, G–J, L–O). Evaluation of GFAP immunofluorescence by relative fluorescence intensity and subsequent statistical analysis showed a main effect of group in the ipsilateral cortex ($F(4,159) = 139.03$, $p < 0.001$), ipsilateral CA2/3 region of the hippocampus ($F(4,159) = 80.64$, $p < 0.001$), and ipsilateral hilus of the dentate gyrus ($F(4,159) = 69.79$, $p < 0.001$). Pairwise comparisons demonstrated that the injury group given vehicle exhibited significantly greater luminance values than those of the two injured groups treated with E2 or the group that received G-1 as well as the uninjured SHAM control group in all regions evaluated (Fig. 5P–R). This robust decrease of GFAP-IR astrocytes observed in the E2 1 mg/kg and G-1-treated injury groups was further reduced in the group

administered 5 mg/kg E2. Taken together, these data suggest that acute post-injury treatment with E2 or G-1 after TBI reduces reactive astrogliosis, a hallmark of the CNS response to trauma.

E2 or G-1 treatment does not alter GPER protein expression in the ipsilateral hippocampus

To evaluate the effect of injury on the expression of GPER in the ipsilateral hippocampus, we used Western blot analysis to quantify the relative amount of GPER protein following TBI (Fig. 6). Representative blots from the uninjured SHAM control group, TBI vehicle-treated group, E2 5 mg-treated group, and G-1-treated group are presented in Figure 6A. Quantification via densitometry followed by subsequent statistical analysis did not demonstrate a main effect for group ($F(3,15) = 1.43$, $p = 0.28$, n.s.). As shown in Figure 6B, normalized GPER protein expression showed a non-significant trend toward reduction in all injury groups compared to the uninjured SHAM control group. We infer from these data that GPER protein expression may be reduced by injury, but the effect is not robust.

Discussion

E2 or G-1 increases cell survival, decreases neuronal degeneration and apoptosis, and decreases astrogliosis

The results of this study provide direct evidence that E2 or G-1 confers protection in adult male rats subjected to LFP brain injury when assessed at 24 hours following TBI. Specifically, we show that an acute bolus dose of E2 or G-1 administered intravenously 1 h post-injury significantly increases neuronal survival in the ipsilateral hippocampus and decreases neuronal degeneration and apoptotic cell death in both the ipsilateral cortex and hippocampus. We also report a significant reduction in astrogliosis. These effects were observed to be chiefly dose-dependent for E2, with the 5 mg/kg dose generating a more robust level of protection. This study combined an acute post-injury drug administration timepoint with an intravenous bolus dose of water-soluble E2 at supraphysiologic levels or an intravenous bolus dose of the specific GPER agonist G-1 in the clinically relevant model of moderate-severe TBI in male rats. Thus, our findings further elucidate estrogenic compounds as a clinically relevant pharmacotherapeutic strategy for treatment of secondary injury following TBI, and intriguingly, reveal a novel potential therapeutic target in GPER. However, the acute timepoint used in this study of 24 h is not sufficient to determine if these effects would persist long-term. Additional studies investigating apoptosis and reactive astrogliosis at longer timepoints are necessary to ascertain the validity of GPER as a pharmacological target for treatment of TBI.

It is well-established that TBI produces necrotic and apoptotic cell death in humans and in rodents subjected to the moderate-severe LFP brain injury model.^{40–43} Thus, we evaluated both neuronal survival and neuronal death in ipsilateral brain structures that are especially vulnerable to significant cell death following LFP brain injury.³⁴ Since the LFP model induces both focal and diffuse injury in cortex, we also assessed the effects of treatment on apoptosis by processing brain tissue for expression of active caspase 3 immunoreactivity, an indicator of late-stage apoptosis.⁴¹ We found that E2 or G-1 treatment reduced apoptosis in both cortex and hippocampus, which is in accordance with previous studies that document attenuation of programmed cell death by the

FIG. 5. Effect of E2 or G-1 administration on reactive astrogliosis in the ipsilateral cortex, CA 2/3 region of the hippocampus, and hilus of the dentate gyrus. Representative micrographs of GFAP+ astrocytes are shown at 400X magnification (cortex, A–E; CA 2/3 region of the hippocampus, F–J; hilus region of the dentate gyrus, K–O; scale bar = 100 μ m) and numbers of dying cells were quantified with relative luminance intensity (P–R). A, F, and K depict the SHAM group, B, G, and L the TBI VEH group, C, H, and M the TBI E2 1 mg group, D, I, and N the TBI E2 5 mg group, and E, J, and O the TBI G1 group. White arrows indicate representative GFAP+ astrocytes (B, G, and L) whose numbers appear to be attenuated in C–E, H–J, and M–O. Quantification of relative fluorescence in arbitrary units (mean scores $\pm$ standard error of the mean) of GFAP+ astrocytes in cortex, the CA 2/3 region of the hippocampus, and the hilus is presented in P, Q, and R, respectively (*significant difference compared to SHAM; +significant difference compared to VEH-treated injury group; **significant difference compared to E2 1 mg-treated injury group; #significant difference compared to E2 5 mg-treated group, $p < 0.001$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol; G1, G-1). Color image is available online at www.liebertpub.com/neu

administration of E2 in this and other models of brain injury.^{25,44–46} In addition, we analyzed protein expression levels of active caspase 3 in the ipsilateral hippocampus and found that the increase in active caspase 3 protein following TBI induction was significantly reduced following G-1 administration, an intriguing finding. The E2 treatment results are in contrast to earlier work that found E2 provided no significant reduction in caspase-3 immunoreactivity in the hippocampal CA3, hilar, and granule cell layers induced by LFP TBI.⁴⁷ However, the Lebesgue study differed substantially in key experimental elements including the use of E2 as a pretreatment at a much lower dose and with less direct route of administration in ovariectomized female rats. The majority of data suggest that E2

does indeed protect against apoptotic cell death, and this is perhaps mediated through mitochondrial anti-apoptotic mechanisms, as some studies investigating estrogen in CNS trauma have reported favorable modulation of Bcl family proteins.^{15,17,20,25,48,49}

Astrogliosis is characterized by hypertrophy of astrocytic processes and upregulation of GFAP expression in astrocytes, features which are both commonly observed following TBI.^{31,50} We found a significant dose-dependent reduction in GFAP-IR staining in both cortex and hippocampus in treatment groups that received E2, similar to previously reported findings.^{51–54} In addition, protein expression levels of GFAP in the ipsilateral cortex following TBI induction were significantly increased; this was attenuated by G-1

FIG. 6. Effect of E2 or G-1 administration on GPER protein expression levels in the ipsilateral hippocampus. Representative bands from Western blots are shown in (A). Quantification of total protein expression relative to α -tubulin in each lane is presented in (B) ($p=0.28$; SHAM, control animals; VEH, vehicle; E2, 17 β -estradiol; G1, G-1; a.u., arbitrary units).

treatment, a novel finding that warrants further investigation (data not shown).

Potential mechanisms of action involved in E2-mediated neuroprotection

The mechanisms through which E2 provides neuroprotection post-TBI have yet to be thoroughly elucidated. It is known that E2 acts on target cells through both genomic and nongenomic pathways, exerting its effects via direct or indirect transcriptional regulation, initiation of intracellular second messenger cascades, and through antioxidant and vascular mechanisms.^{20,28,30,55–57} Receptors involved in mediating the effects of E2 include the classical nuclear receptors ER α and ER β , membrane-associated splice variants of ERs, and the recently characterized GPER.^{28,30,58} Briefly, ER α and ER β are ligand-activated transcription factors that bind E2 and interact with co-factors at the promoter of target genes to stimulate or suppress transcription.⁵⁶ GPER, located chiefly in the membrane of the endoplasmic reticulum, binds E2 and stimulates downstream signaling events via G $\beta\gamma$ -mediated transactivation of the epidermal growth factor receptor (EGFR), which initiates crucial kinase cascades such as MAPK and PI3K/Akt and instigates rapid elevation of intracellular Ca²⁺.^{20,30} Activation of MAPK and PI3K signaling pathways is of particular interest as a number of survival, proliferation, and growth-related factors are regulated downstream of the PI3K/Akt signaling pathway, suggesting this pathway may be involved in mediating E2's neuro- and cellular protection. Previous research has implicated the involvement of ER α and the PI3K/Akt signaling pathway in mediating protection afforded by E2 administration, but the contribution of GPER following TBI remains uninvestigated.^{18,20,44,45,59–61}

Little is known of the function of GPER in the brain or how brain trauma may alter this receptor's activity. It has been reported that GPER is expressed in the CNS of both adult male and female rats,

and is found throughout the brain, including the hippocampal formation.^{62–63} GPER activation has been implicated in cognitive performance. For example, treatment of female rats with G-1 was found to reverse ovariectomy-induced memory deficits in a delayed-matching-to-position T-maze task, perhaps by enhancing hippocampal cholinergic functioning, as sustained administration of G-1 caused an increase in potassium-stimulated acetylcholine release.^{64,65} In addition, GPER may be involved in modulation of mood disorders. It has been reported that G-1 treatment attenuated serotonin 1A (5HT-1A) receptor signaling in the rat hypothalamus.⁶⁶ The MAPK signaling pathway has been implicated as a mechanism of GPER action: a previous study indicated that treatment of trigeminal ganglion cells with G-1 induced activation of the MAPK signaling pathway *in vitro* and also increased facial allodynia *in vivo*.⁶⁷ This activation of ERK 1/2 was also implicated in neuroprotection against excitotoxicity in cultured cortical neurons treated with G-1 following exposure to glutamate, an effect that was significantly reduced with knockdown of GPER via the introduction of shRNAs.⁶⁸ Additionally, another group reported that in immortalized hippocampal cell lines, G-1 pretreatment for 1 hour significantly reduced cell death caused by glutamate excitotoxicity, and this protection was reversed with application of the GPER-specific antagonist G15.⁶⁰ Recently, research focused on the effects of G-1 on astrocytic glutamate transporters, involved in mediating neuroprotective effects of E2 in models of excitotoxic cell death, found that treatment with G-1 increased expression of glutamate transporter-1 (GLT-1), prevented manganese-induced reduction in expression of GLT-1 protein and uptake of glutamate in cultured astrocytes, and linked this effect to both MAPK and PI3K signaling pathways.⁶⁹ GPER agonism by G-1 has also been reported to reduce hippocampal CA1 cell death significantly following global ischemia in middle-aged ovariectomized female rats and to significantly attenuate cell death due to oxidative stress in primary neuronal cultures while upregulating the expression of phosphorylated ERK 1/2, bcl-2, and pro-caspase 3.^{70,71} In a mouse model of Parkinson's disease, G-1 administration was reported to exert protection equivalent to that of E2 against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in the striatum and substantia nigra, which was blocked by the addition of G15.⁷²

Although evidence to date suggests that GPER plays a protective role in brain, no studies have yet investigated its involvement in attenuation of cell death following TBI. This study demonstrates the acute protective potential of GPER activation post-TBI and may represent a promising new drug target for treatment of TBI; however, additional subacute and long-term time points need to be examined to determine the full impact of GPER activation as a protective agent, as our study only investigated the effects of G-1 administration at 24 hours post-injury. That caveat aside, the possible contribution of GPER in E2-mediated protection in the CNS after trauma is intriguing and merits future study.

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Author Disclosure Statement

The authors declare no competing financial interests.

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