Restoration of Neuroendocrine Stress Response by Glucocorticoid Receptor or GABA_A Receptor Antagonists after Experimental Traumatic Brain Injury

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Abstract

We previously reported that traumatic brain injury (TBI) produced by moderate controlled cortical impact (CCI) attenuates the stress response of the hypothalamic-pituitary-adrenal (HPA) axis between 21 and 70 days postinjury and enhances the sensitivity of the stress response to glucocorticoid negative feedback. In the current study, we investigated two possible mechanisms for the CCI-induced attenuation of the HPA stress response—i.e., glucocorticoid receptor (GR) and GABA-mediated inhibition of the HPA axis, with the GR antagonist, mifepristone (RU486), or the GABA(A)-receptor antagonist, bicuculline. In addition, we examined the effect of moderate CCI on GR and inhibitory neurons histologically in subfields of the hippocampus, medial prefrontal cortex, and amygdala. We show that at 30-min after onset of restraint stress, GR as well as GABA antagonism with MIFE or BIC, respectively, reversed the attenuating effects of moderate CCI on the stress-induced HPA response. Our histological results demonstrate that moderate CCI led to a loss of glutamic acid decarboxylase 67 or parvalbumin-positive inhibitory neurons within regions of the hippocampus and amygdala but did not lead to significant increases in GR in these regions. These findings indicate that suppression of the stress-induced HPA response after moderate CCI is mediated by the inhibitory actions of both GR and GABA, with a corresponding loss of inhibitory neurons within brain regions with neural pathways affecting limbic stress-integrative pathways.

Key words: bicuculline; corticosterone; glutamic acid decarboxylase 67; mifepristone; parvalbumin

Introduction

ALTERED ACTIVITY OF THE hypothalamic-pituitary-adrenal (HPA) axis after traumatic brain injury (TBI) has been demonstrated both clinically^{1–4} and experimentally.^{5–9} While these reports all point to baseline neuroendocrine dysfunction after TBI, we previously demonstrated that TBI, produced by moderate lateral cortical contusion injury (CCI) in rodents, causes long-term dysregulation of the neuroendocrine stress response.^{10,11} We observed attenuation of the HPA stress response for as long as 70 days after moderate CCI, an effect that appears to be mediated by enhanced glucocorticoid negative feedback control of the HPA axis.¹²

Given the important role of stress coping behavior in the long-term neurobehavioral outcomes of brain injury, further investigation of the dysregulated neuroendocrine stress response after moderate CCI is clearly warranted. Termination of the HPA stress response is mediated by glucocorticoid negative feedback,

and glucocorticoid (and mineralocorticoid) receptors are abundantly expressed in the forebrain—i.e., hippocampus, prefrontal cortex, and amygdala.^{13–15} Our finding of enhanced sensitivity to the synthetic glucocorticoid, dexamethasone, after moderate CCI, indicates that glucocorticoid receptor-rich regions in these forebrain structures may have been impacted by our CCI.¹² Moreover, limbic and cortical regions are themselves stress responsive and activate or inhibit the HPA axis by their respective glutamatergic or GABAergic inputs to the hypothalamus.¹⁶

We therefore sought to investigate two possible mechanisms for the CCI-induced attenuation of the HPA stress response—i.e., glucocorticoid receptor (GR) and GABA-mediated inhibition of the HPA axis, with the GR antagonist, mifepristone (RU486), or the GABA_A-receptor antagonist, bicuculline. In addition, we examined the effect of moderate CCI on GR and inhibitory neurons histologically in subfields of the hippocampus, medial prefrontal cortex, and amygdala.

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Methods

Subjects

Individually caged adult male Sprague Dawley rats from Charles River Breeding Labs (Hollister, CA) were maintained in our standard temperature and lighting conditions ($22 \pm 1^\circ\text{C}$, 14 h/10 h lighting cycle, lights on: 04:00–18:00). All experimental procedures were approved by the UCLA Institutional Animal Care and Use Committee.

Surgical procedures

At 60–70 days of age, animals were anesthetized with isoflurane (2.0–2.5% in 100% O_2 , 2.0 mL/min flow rate) and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the head positioned in a horizontal plane with respect to the interaural line. During all surgical procedures, body temperature was maintained at $37\text{--}38^\circ\text{C}$ using a thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA). All surgical procedures were performed under aseptic conditions and have been previously described in detail.^{10–12,17,18}

In brief, after a midline incision, the skin, fascia, and temporal muscle were reflected. Animals receiving CCI were subjected to a 6-mm diameter craniotomy over the left parietal cortex centered at 3 mm posterior and 4 mm lateral to bregma. An electronically controlled, small bore, dual-stroke, pneumatic piston cylinder with a 40-mm stroke (Hydraulics Control, Inc., Emeryville, CA) was mounted onto a stereotaxic micromanipulator (Trentwells Co., Coulterville, CA), allowing for precise control of the impact site and depth of tissue compression. The piston cylinder was angled 22.0° degrees away from vertical, enabling the flat, circular impactor tip (5 mm diameter) to be perpendicular to the surface of the brain at the site of injury. After induction of a moderate CCI (30 psi or $\sim 2.8\text{ m/sec}$, 2.0 mm depth), the fascia and scalp were sutured closed, and triple antibiotic was applied to the wound margins. Sham-operated control rats underwent similar procedures to control for surgical stress and duration of anesthesia, but did not receive craniotomy or any impact. Animals were returned to their cages after recovery from anesthesia.

Drug treatment

In experiment 1, 15 to 19 days after injury, 16 CCI and 17 sham animals were matched by body weight and were divided into two groups that were administered a single daily injection of the GR antagonist mifepristone (MIFE; Sigma-Aldrich, 10 mg/kg subcutaneously dissolved in propylene glycol with 1% ethanol) or the propylene glycol/ethanol (PPG) vehicle for 5 days.¹⁹ On the fifth day, 60-min after the last injection, animals were exposed to restraint stress as described below. The treatment time-frame was based on our previous demonstration of hypofunction of the HPA stress response by 21 days after moderate CCI¹⁰ and the 1 h delay between treatment and restraint was based on the protocol of Wulsin and associates.¹⁹

In experiment 2, 28 days after injury, 18 CCI and 18 sham animals were matched by body weight and were divided into two groups that were administered the GABA_A-receptor antagonist (+)-bicuculline (BIC; Sigma-Aldrich, dissolved in 0.1 N HCl and pH adjusted to 7.0 with 0.1 N NaOH, diluted to 4.0 mg/kg with saline for intraperitoneal [i.p.] injection) or saline (SAL) at 5 min before restraint stress, based on the protocol of Beleslin and colleagues.²⁰

In experiment 3, at 57–59 days after injury, 6 CCI and 6 sham injured animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused with phosphate buffered saline (PBS, 0.1 M) followed by 4% paraformaldehyde in 0.1 M PBS. The fixed brains were extracted from the skull and post-fixed for 1 h in the paraformaldehyde solution at 4°C . Each brain was then frozen

and sectioned in the coronal plane (40- μm thick) on a sliding microtome (American Optical, Model 860, Buffalo, NY), saving six adjacent tissue sections every 500- μm into an antifreeze solution (4.36 g Na_2HPO_4 and 1.25 g Na_2HPO_4 in 320 mL H_2O , 240 mL ethylene glycol, and 240 mL glycerol). These sections were maintained at -20°C until processed for immunohistochemistry (IHC), as described below.

Restraint

At 60 min after MIFE/PPG administration (experiment 1) or at 5 min after BIC/SAL administration (experiment 2), restraint stress was initiated. Animals were placed into flat-bottom Plexiglas restraining tubes (13 cm $\times$ 6 cm $\times$ 8 cm, Harvard Apparatus Co.) and tightly restrained for 30 min followed by light restraint for an additional 60 min. A blood sample was obtained by tail venipuncture immediately before onset of restraint in both experiment 1 and experiment 2. Additional blood samples were obtained at 30 and 90 min after stress onset in both experiments. All testing occurred between 0900 and 1300 h.

Assay of plasma content of corticosterone (CORT)

Blood samples (300 μL) were collected at the time points described above, in microcapillary blood collection tubes that contained ethylenediaminetetraacetic acid with added aprotinin (200 KIU/mL). After centrifugation at 2000 rpm for 20 min, plasma was separated and stored at -80°C until analysis for CORT.

Plasma CORT was assessed with a rat CORT ^{125}I radioimmunoassay kit (MP Biomedicals, Irvine, CA), as we have done previously.¹⁸ The reported detection limit of this assay is 8 ng/mL, the recovery of exogenous CORT is 100%, and intra- and interassay coefficients of variation are lower than 10.3% and 7.2%, respectively. The results are expressed as nanograms per milliliter of plasma.

Immunohistochemistry for glutamic acid decarboxylase 67 (GAD-67), parvalbumin (PV), and GR

At the time IHC was performed, 4–6 tissue sections containing the regions of interest (medial prefrontal cortex, hippocampus, and amygdala) were selected for each animal, and the IHC reactions were conducted simultaneously on tissues from all rats in each group. Anatomical nomenclature and anterior-posterior (mm from bregma) location of brain regions analyzed are consistent with those from the rat brain atlas of Paxinos and Watson.²¹

Before staining for the GABA-synthesizing enzyme GAD-67, selected tissue sections from each region were removed from the antifreeze solution and placed into 0.1 M PBS at 4°C overnight. Sections were then rinsed $3 \times 10\text{ min}$ in fresh PBS at room temperature, and endogenous peroxidase activity was quenched by incubating sections in 0.3% H_2O_2 in dH_2O for 30 min. Sections were placed into 1% NaBH_4 in dH_2O for 15 min, washed $3 \times 10\text{ min}$ in 0.1 M PBS and then blocked with 10% normal horse serum for 30 min at room temperature. Primary antibody (anti-GAD-67, clone 1G10.2, Millipore, Temecula, CA) was diluted 1:2000 by 0.1 M PBS with 3% normal goat serum, and tissue sections were incubated for 48 h at 4°C . After another PBS rinse, sections were moved to the secondary antibody (biotinylated horse anti-mouse IgG, PK-6102, Vector Laboratory) that was diluted 1:1000 in 0.1 M PBS with 3% normal horse serum for 2 h. Sections were rinsed in PBS, incubated in avidin conjugated to HRP (Vector ABC kits) for 1 h, rinsed in PBS, and the primary antibody was visualized by exposing sections to 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 in PBS for 3 min. Visualization was stopped by $3 \times 10\text{ min}$ washes in PBS, the free floating sections were mounted onto gelatinized slides, and tissue was air dried overnight at room

temperature. Mounted sections were dehydrated in ethanol, cleared in Hemo-D, and cover-slipped using Cytoseal 60.

Additional tissue sections were processed to label GABAergic cells containing the calcium-binding protein PV. These sections were removed from the antifreeze solution and placed into 0.1 M Tris buffered saline (TBS) at 4°C overnight. Sections were then rinsed 3 × 10 min in fresh TBS at room temperature and then blocked with 3% normal horse serum in TBS containing 0.1% Triton X-100 (TBST) for 1 h. Primary antibody (anti-parvalbumin, MAB1572, Millipore, Temecula, CA) was diluted 1:10000 by 0.1 M TBST, and tissue sections were incubated for 24 h at room temperature. After rinsing 3 × 10 min in 0.1 M TBS, sections were moved to the secondary antibody (biotinylated horse anti-mouse IgG, PK-6102, Vector Laboratory) that was diluted 1:700 in 0.1 M TBS with 3% normal horse serum for 30 min. After rinsing in 0.1 M TBS, sections were incubated in avidin conjugated to HRP (Vector ABC kits) for 1 h, rinsed in TBS, and the primary antibody was visualized by exposing sections to 0.05% DAB, 0.01% H₂O₂, and 0.4% nickel chloride in PBS for 2 min. Visualization was stopped by 3 × 10 min rinses in TBS, the free floating sections were mounted on slides, air dried, dehydrated, cleared, and cover-slipped as described above.

After removal from antifreeze solution, sections selected for GR staining were maintained at 0.1 M PBS at 4°C. Sections were rinsed 3 × 10 min in fresh PBS at room temperature, incubated for 1 h in 50% methanol and 0.2% H₂O₂, and then blocked with 3% normal goat serum in PBS containing 0.1% Triton X-100 (PBST) for 1 h. The primary GR-antibody (sc-8992, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was diluted 1:1500 in the blocking solution, and tissue sections were incubated for 24 h at 4°C. After rinsing 3 × 5 min in 0.01 M PBS, sections were moved to the secondary antibody (biotinylated anti-rabbit IgG, BA-1000, Vector Laboratory), diluted 1:200 in 0.01 M PBS with 5% normal goat serum, for 30 min. Sections were rinsed 3 × 5 min in 0.01 M PBST, incubated in avidin conjugated to HRP (Vector ABC kits) for 1 h, rinsed 2 × 5 min in PBST followed by 2 × 5 min in acetate buffer (pH 6.0), and the primary antibody was visualized by exposing sections for 7 min to a Ni-DAB chromagen solution (2.0% nickelous ammonium sulfate, 0.035% DAB, 0.006% H₂O₂ in acetate buffer), dehydrated, cleared, and cover-slipped as described above.

Microscopy and cell counting. Preliminary microscopic examinations of each GAD-67 and PV IHC-stained brain were conducted to choose appropriately matched sections from each rat containing two anterior-posterior levels within each region of interest. For the medial prefrontal cortex, GAD-67 and PV-positive neurons were counted within the midline cingulate (Cg1), pre-imbic (PrL), and infralimbic (IL) regions at 3.2 and 2.7 mm anterior to bregma. For the amygdala, GAD-67 and PV-positive neurons were counted within the basolateral (BLA), central (CeA), and medial (MeA) nuclei at −2.3 and −2.8 mm from bregma, and for the dorsal hippocampus these neurons were counted in the CA1, CA3, and the hilar region of the dentate gyrus at −3.2 and −3.7 mm from bregma.

Cell counts in left and right hemispheres were performed using bright-field illumination with a Leica microscope (Leica Microsystems GmbH, Model DMRE) that was interfaced with a computer and Stereo Investigator software (MicroBrightField Inc., Colchester, VT). In each case, the tissue was first visualized using the 2.5× objective, and the regions of interest were outlined (contoured) using the imaging software. GAD-67- or PV-positive neurons lying within the areas of each contour are counted using 20× and 40× objectives. The order in which cell counts for individual animals were performed for each region and for each stain was randomized, with the person conducting the cell counts remaining blinded to the experimental treatment group until all data were collected.

For each brain region, the areas and cell counts from both anterior-posterior planes were summated, and cell density

measures (cells/mm²) were calculated for each animal. To control for potential differences in anterior-posterior planes used for cell counting, after first verifying no injury effects on cell densities contralateral to injury, the final group data for each brain region are expressed as the mean percent ($\pm$ standard error of the mean [SEM]) cell density in the left (injured) hemisphere relative to the right (uninjured) hemisphere.

Image analysis of GR. Images of all GR-stained tissue sections for each rat were digitally captured at 8 bit, 600 dpi resolution using a flat-bed scanner. To quantify the GR IHC, optical density measures of staining were obtained bilaterally from four different levels of the medial prefrontal cortex (encompassing the Cg1, PrL, and IL regions), three sections containing the dorsal hippocampus (separate measures for the CA1, CA3, and the hilar region of the dentate gyrus), and three tissue sections containing the amygdala (separate measures for the BLA, CeA, and MeA nuclei) using ImageJ software (version 1.45i: National Institutes of Health, Bethesda, MD). Background measures of immunoreactivity were obtained from the optic tract. Optical density values in each region measured were normalized to this remote white matter region using the formula (region intensity-remote intensity)/remote intensity × 100) as previously described.²² Regional optical density values for each animal were calculated by averaging all values in each hemisphere, with these values used to calculate group means per region.

Statistical analysis

Analyses of variance (ANOVA) were performed on the body weight and CORT data for the factors of injury condition (CCI/sham) and drug treatment. Given that body weights varied considerably at the onset of MIFE treatment, all results are expressed as the % change in weight—i.e., difference from treatment onset at each subsequent day. In view of the marked differences in CORT levels in experiment 1 as well as the lesser differences in CORT levels in experiment 2, all results are expressed as the CORT responses—i.e., difference from 0-time at each subsequent time point. Individual group means were compared using *t* tests under the Tukey-Fisher least significant difference (LSD) criterion with α set at 0.05 (two-sided). The IHC data for sham and CCI groups were analyzed for each brain region and stain using unpaired Students *t* tests, with α set at 0.05 (two-sided).

Results

Experiment 1

Body weights before injection of MIFE or PPG did not differ among the groups and averaged 417.15 $\pm$ 6.17 (SEM) g. Repeated measures ANOVA of daily % change in weight over the 5 days of MIFE/PPG injection indicated a significant effect of drug treatment [$F(1,29)=4.45$, $p=0.0436$], but not injury condition nor their interaction (Fig. 1). By day 5, weight gain of the CCI-MIFE group was significantly ($p=0.0213$) less than that of the CCI-PPG group (Fig. 1).

ANOVA indicated that pre-restraint levels of CORT at 60 min after the fifth injection of MIFE/PPG were significantly affected by the drug treatment [$F(1,29)=10.63$, $p=0.0028$] but not by injury condition, nor was there an interaction of the two factors. At this time, CORT levels were significantly ($p=0.02$) higher in the CCI-PPG group than in all other groups (Table 1). Given the differences in the pre-restraint levels of CORT, the effects of restraint are presented as the CORT responses—i.e. differences between the post- and pre-restraint individual levels (Fig. 2).

Restraint significantly increased CORT levels at the 30-min time point in all groups. ANOVAs indicated a significant effect of injury

FIG. 1. Effect of mifepristone (MIFE) or the propylene glycol (PPG) vehicle on the % change in body weight (mean $\pm$ standard error of the mean) from the first day of a 5-day injection period commencing at 15 days after moderate controlled cortical impact (CCI) or sham injury. a: $p=0.0213$ for groups with the same letter. Parentheses indicate number of subjects.

condition ($F[1,29]=8.34$, $p=0.0073$), no effect of drug treatment, but a significant interaction of the two factors ($F[1,29]=7.36$, $p=0.01$) for CORT responses at 30 min after restraint onset. At 90 min after stress onset, CORT responses showed significant effects of injury condition and treatment ($F[1,29]=4.43$, $p=0.0441$ and 8.19 , $p=0.0077$, respectively) but not their interaction (Fig. 2). At both time points, the robust CORT responses of the CCI rats treated with MIFE were similar to those of the sham-MIFE group. As expected, based on previous results,^{10,11} the CORT response of the CCI-PPG group was significantly less than that of the sham-PPGs at both 30- ($p=0.001$) and 90-min ($p<0.05$); however, the contribution of the significantly elevated basal CORT level to this effect cannot be excluded.

Experiment 2

Body weights were similar in all groups (415.54 ± 4.79 g) before injection of BIC. ANOVA indicated a significant effect of injury condition, but not drug treatment or their interaction, on pre-restraint levels of CORT 5 min after the injection of BIC/SAL ($F[1,31]=17.32$, $p=0.0002$). CORT levels were significantly ($p<0.01$) higher at 5 min after injection of SAL in the CCI group

TABLE 1. PRE-RESTRAINT LEVELS (NG/ML) OF PLASMA CORTICOSTERONE (MEAN $\pm$ STANDARD ERROR OF THE MEAN) AT 60 MIN AFTER THE FIFTH SUBCUTANEOUS INJECTION OF MIFEPRISTONE/PROPYLENE GLYCOL AND AT 5 MIN AFTER INTRAPERITONEAL INJECTION OF BICUCULLINE/SALINE AT 19 AND 28 DAYS, RESPECTIVELY, AFTER CONTROLLED CORTICAL IMPACT OR SHAM INJURY

Treatment	Group	
	CCI	Sham
MIFE	81.04 ± 31.57 (9)	142.54 ± 32.09 (9)
PPG	276.46 ± 64.12^a (7)	90.83 ± 22.83 (8)
BIC	28.77 ± 3.63 (9)	20.69 ± 3.55 (9)
SAL	36.55 ± 4.40^b (8)	16.58 ± 1.35 (9)

CCI, controlled cortical impact; MIFE, mifepristone; PPG, propylene glycol; BIC, bicuculline; SAL, saline.

^a $p=0.02$ vs. CCI-MIFE, sham-MIFE, and sham-PPG.

^b $p<0.01$ vs. sham-SAL.

Parentheses indicate number of subjects.

FIG. 2. Effect of mifepristone (MIFE) or the propylene glycol (PPG) vehicle on the corticosterone response to 30-min restraint stress at 19 days after moderate controlled cortical impact (CCI) or sham injury. Corticosterone responses (mean $\pm$ standard error of the mean) in tail vein samples collected at 30 and 90 min after restraint onset represent differences from basal values obtained in tail vein samples collected immediately before restraint at 60 min after the fifth daily subcutaneous injection of MIFE or PPG. a, b, c: $p<0.01$, d: $p<0.05$ for groups with the same letter. Parentheses indicate number of subjects.

than in the sham group (Table 1). Given these differences in the pre-restraint levels of CORT, the effects of restraint are presented as the CORT responses—i.e., differences between the post- and pre-restraint individual levels (Fig. 3).

At 30 min after stress onset, ANOVA indicated an effect of injury condition ($F[1,29]=11.88$, $p=0.0018$), but not drug treatment or the interaction of the two factors. At this time, the CORT response in the presence of SAL was significantly ($p=0.001$) lower in the CCI rats than in the sham group; BIC abolished this effect. At 90 min, CORT responses were similar in all groups.

Experiment 3

There was no effect of CCI on the measures of cell densities of either GAD-67-positive or PV-positive inhibitory neurons within

FIG. 3. Effect of bicuculline (BIC) or saline (SAL) vehicle on the corticosterone response to 30-min restraint stress at 28 days after moderate controlled cortical impact (CCI) or sham injury. Corticosterone responses (mean $\pm$ standard error of the mean) in tail vein samples collected at 30 and 90 min after restraint onset represent differences from basal values obtained in tail vein samples collected immediately before restraint at 5 min after intraperitoneal injection of BIC or PPG. a: $p=0.001$ for the groups with the same letter. Parentheses indicate number of subjects.

TABLE 2. LEFT HEMISPHERE CELL DENSITIES EXPRESSED AS A PERCENT OF CELL DENSITIES IN THE RIGHT HEMISPHERE (MEAN $\pm$ STANDARD ERROR OF THE MEAN) FOR EACH BRAIN REGION STAINED FOR GLUTAMIC ACID DECARBOXYLASE-67 OR PARVALBUMIN IN RATS WITH CONTROLLED CORTICAL IMPACT OR SHAM INJURY

	GAD-67		Parvalbumin	
	Sham	CCI	Sham	CCI
Medical prefrontal cortex				
Cg1	104.9 $\pm$ 2.9%	99.4 $\pm$ 2.1%	98.0 $\pm$ 2.9%	93.2 $\pm$ 4.0%
PrL	96.7 $\pm$ 4.0%	94.6 $\pm$ 2.9%	93.7 $\pm$ 2.5%	97.5 $\pm$ 3.1%
IL	94.6 $\pm$ 5.9%	91.5 $\pm$ 3.9%	91.0 $\pm$ 4.0%	95.6 $\pm$ 3.6%
Dorsal hippocampus				
CA1	101.2 $\pm$ 0.9%	89.3 $\pm$ 3.5% ^b	101.9 $\pm$ 6.8%	79.3 $\pm$ 6.2% ^a
CA3	95.4 $\pm$ 2.7%	91.6 $\pm$ 7.4%	100.3 $\pm$ 4.6%	79.8 $\pm$ 8.4%
Hilus	100.8 $\pm$ 1.6%	81.4 $\pm$ 3.7% ^c	93.0 $\pm$ 4.4%	57.0 $\pm$ 8.1% ^b
Amygdala				
BLA	97.8 $\pm$ 2.0%	77.4 $\pm$ 2.8% ^c	101.5 $\pm$ 3.2%	94.4 $\pm$ 5.0%
CeA	97.4 $\pm$ 2.6%	86.3 $\pm$ 7.5%	97.3 $\pm$ 2.6%	97.1 $\pm$ 2.6%
MeA	100.7 $\pm$ 4.2%	78.6 $\pm$ 4.3% ^b	95.1 $\pm$ 4.4%	79.9 $\pm$ 7.1%

GAD, glutamic acid decarboxylase; CCI, controlled cortical impact; Cg1, midline cingulate; PrL, prelimbic; IL, infralimbic; BLA, basolateral; CeA, central; MeA, medial.

^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. sham.

Cg1, PrL, or IL regions of the medial prefrontal cortex at 2 months post-injury (Table 2).

In the CA1 region, there was a significant CCI-induced loss of GAD-67-positive ($p < 0.01$) and PV-positive ($p < 0.05$) neurons. For the CA3 region, there was a trend for PV-positive cell density measures being reduced in the CCI group compared with sham ($p = 0.057$), but this trend was not seen for GAD-67-positive neurons in the CA3 region. Loss of GAD-67-positive ($p < 0.001$) and PV-positive ($p < 0.01$) neurons in the ipsilateral dentate hilus region of CCI rats was also found in these cell density measures compared with sham (Table 2).

For the BLA nucleus of the amygdala, there was a significant loss of GAD-67 neurons ipsilateral to moderate CCI relative to

TABLE 3. REGIONAL OPTICAL DENSITY VALUES FOR GLUCOCORTICOID RECEPTOR IMMUNOHISTOCHEMISTRY (MEAN $\pm$ STANDARD ERROR OF THE MEAN) IN LEFT AND RIGHT HEMISPHERES IN RATS SURVIVING 57–59 DAYS AFTER LATERAL (LEFT) CONTROLLED CORTICAL IMPACT OR SHAM INJURY

	Left		Right	
	Sham	CCI	Sham	CCI
Medial prefrontal cortex				
	55.1 $\pm$ 4.0	52.1 $\pm$ 4.3	57.8 $\pm$ 4.2	54.6 $\pm$ 4.7
Dorsal hippocampus				
CA1	86.8 $\pm$ 3.5	102.7 $\pm$ 6.9	87.0 $\pm$ 4.5	94.0 $\pm$ 6.6
CA3	80.3 $\pm$ 4.5	80.4 $\pm$ 5.1	80.2 $\pm$ 3.6	82.2 $\pm$ 4.6
Hilus	79.9 $\pm$ 9.2	79.6 $\pm$ 12.9	80.9 $\pm$ 9.3	78.4 $\pm$ 10.7
Amygdala				
BLA	207.1 $\pm$ 7.6	183.1 $\pm$ 17.3	211.5 $\pm$ 9.3	187.5 $\pm$ 17.0
CeA	226.2 $\pm$ 5.6	205.8 $\pm$ 19.8	227.3 $\pm$ 9.7	209.9 $\pm$ 20.2
MeA	235.1 $\pm$ 11.9	212.3 $\pm$ 20.1	232.9 $\pm$ 11.0	213.2 $\pm$ 22.3

BLA, basolateral; CCI, controlled cortical impact; CeA, central; MeA, medial.

sham injury ($p < 0.001$), with no significant reduction of PV-positive cells in this nucleus after CCI. Although a mild loss of GAD-67-positive cells in the CeA nucleus was observed in the CCI group, no significant injury effects were found for this region (Table 2). Left MeA cell density as a percent of right cell density for GAD-67-positive neurons was also significantly reduced in the CCI group compared with sham ($p < 0.01$), while the reduced cell densities for PV-positive neurons in the MeA of CCI animals only showed a trend toward significance ($p = 0.097$).

Data for the optical density measures of GR IHC are shown in Table 3. The lowest optical density values were observed in the medial prefrontal cortex, and there were no significant effects of injury condition on these values in the left or right hemisphere. The optical density values were somewhat higher in the hippocampal regions, but there were no significant injury effects for any region in either hemisphere. The only region showing a trend toward a significant injury effect was the CA1, where values in the left CA1 of CCI animals were increased relative to optical densities in sham controls ($p = 0.068$). The highest optical density values for GR IHC were observed in the nuclei of the amygdala, but there were also no significant effects of injury on these measures in any region or hemisphere.

Discussion

The current study was initiated to explore mechanisms that might explain our previous reports that moderate CCI attenuates the HPA stress response between 21 and 70 days post injury^{10,11} and enhances the sensitivity of the stress response to glucocorticoid negative feedback.¹² We show that at 30 min after onset of restraint stress, GR as well as GABA antagonism with MIFE or BIC, respectively, reverses the attenuating effects of CCI on stress-induced HPA activity. These findings indicate that CCI suppression of the stress-induced HPA response is mediated by the inhibitory actions of both GR and GABA.

We then proceeded to determine whether moderate CCI would lead to increases in GR and/or loss of inhibitory neurons within brain regions with neural pathways affecting limbic stress-integrative pathways. Our current results indicate that moderate CCI did not lead to significant changes in GR nor in either GAD-67- or PV-positive inhibitory neurons in regions of the medial prefrontal cortex; however, in contrast to no effects on hippocampal GR, CCI did lead to significant loss of inhibitory neurons in the CA1 and hilar regions of the ipsilateral hippocampus. Moderate CCI also led to significant loss of GAD-67-positive neurons in the BLA as well as in the MeA, but no effect on GR in these regions. Thus, at least for the hippocampus and amygdala, these results indicate that moderate CCI leads to loss of inhibitory neurons within brain regions with neural pathways affecting limbic stress-integrative pathways.

MIFE reduced body weight gain of CCI rats over the 5 treatment days and lowered basal CORT levels relative to CCI-PPG controls at 60 min after the last injection. In contrast, body weight and basal CORT levels were unaffected in sham injured rats. Using the same treatment paradigm, Wulsin and coworkers¹⁹ reported no effect on basal CORT levels after 5 days of MIFE in intact rats. Nevertheless, it should be noted that basal levels of CORT at 60 min after the fifth daily injection with either MIFE or the PPG vehicle were elevated compared with basal levels at 5 min after one injection of BIC/Sal (Table 1). The CCI-induced attenuation of the CORT response after restraint stress observed in vehicle treated sham injured controls, similar to our previous reports,^{10,11} was prevented by MIFE

treatment after CCI. This effect of MIFE is indicative of a decrease in GR-mediated feedback efficacy. Not finding any significant CCI-associated increases in GR receptors *per se* in regions of the medial prefrontal cortex, amygdala, and hippocampus, however, our data suggest that restoration of the stress-induced CORT response by MIFE was because of its effect on limbic stress-integrative pathways to the periventricular nucleus (PVN), as discussed below.

Numerous studies have demonstrated that forebrain GRs are essential for negative feedback regulation of the HPA axis—in particular, glucocorticoid feedback inhibition of acute psychogenic stress responses.²³ The rapid action of glucocorticoids has been shown to be triggered by the activation of membrane-associated receptors and nongenomic signaling mechanisms.^{24–26} Evidence indicates that glucocorticoids activate divergent G-protein signaling pathways that act in a synapse-specific manner to suppress excitatory synaptic glutamate inputs and facilitate inhibitory synaptic GABA inputs to magnocellular²⁷ as well as parvocellular²⁸ neurons of the PVN. Whereas all rats in experiment 2 showed a marked CORT response to restraint stress, at 30 min after onset of restraint stress, the CORT responses of the BIC-treated CCI and sham groups did not differ while the CORT responses of the SAL-injected CCI group were significantly lower compared with the responses of SAL-injected shams.

In pilot studies, we found that lower doses of BIC (0.8 and 1.5 mg/kg) given 15 min before stress onset (data not shown; cf. Guo and associates²⁹) did not interfere with the expected CCI-induced attenuation of the stress response; however, the higher dose reported here given 5 min before stress (cf. Beleslin and colleagues²⁰) prevented attenuation of the stress response and did not produce any overt signs of seizures. Thus, restoration of the stress-induced CORT response with BIC indicates that CCI may have impacted GABAergic PVN projecting neurons from upstream limbic or cortical regions that are stress responsive and regulate the HPA axis—e.g., ventral subiculum, medial prefrontal cortex, amygdaloid nuclei, and lateral septum.¹⁶

Whereas GAD-67 levels have been shown to be increased within the medial prefrontal cortex from 1 to 28 days post-injury³⁰ and to return to control levels by 4 months post-CCI,³¹ our current results for PV- and GAD-67-positive cells in the regions of the prefrontal cortex, showing no effects at 57–59 days after moderate CCI, indicate that changes in numbers of inhibitory cells within Cg1, PrL, or IL are not likely to explain the altered HPA axis responses seen after TBI.

The hippocampus sends excitatory inputs to numerous brain regions containing GABAergic cells that project to the PVN.^{16,32} Thus, the transsynaptic inhibition of the PVN arising from the hippocampus could be increased if there is a loss of inhibitory cells within the hippocampus. Although increased levels of GAD-67 within the hippocampus have been reported at 14 days after CCI,³⁰ several studies have reported loss of inhibitory neurons, alterations in GABA receptor subunits, and/or dysfunctional inhibitory synapses resulting in increased excitability within hippocampal regions at weeks to months after experimental TBI.^{33–37} Current results, showing loss of inhibitory neurons within hippocampal regions at 57–59 days after moderate CCI, could conceivably lead to increased excitatory drive of surviving glutamatergic neurons of the hippocampus, leading to greater inhibition of the HPA response to stress as occurs after moderate CCI.

In contrast to the prefrontal cortex and hippocampus, the amygdala is thought to normally provide a stimulatory drive to the HPA axis.^{16,32} MeA inhibitory neurons project to several regions containing GABAergic cells that, in turn, project to the PVN (with potential to increase HPA activity). Thus, the HPA response could

be reduced if there is a loss of inhibitory cells within the MeA, as was found after moderate CCI in the current study (Table 2). A similar arrangement for transsynaptic disinhibition of the PVN and HPA activity exists for the CeA, although no significant cell loss in this nucleus after moderate CCI was found in the current study (Table 2). Finally, excitatory cells in the BLA project to the MeA and CeA and to the bed nucleus of the stria terminalis (BST).^{16,32} Loss of inhibitory cells in the BLA, with increased excitation of GABAergic cells in the MeA and CeA, could increase HPA activity. Conversely, loss of inhibition of glutamatergic cells in the BLA projecting to the BST could increase inhibitory drive from the BST to the PVN. Based on the current results, either of these results from loss of inhibitory cells in the amygdala is likely to occur after moderate CCI, because this group had loss of GAD-67-positive cells in both the BLA and MeA.

Evidence is accumulating for an association between physical and psychological brain injuries, such as post-traumatic stress disorder (PTSD) and depression, among other psychiatric disorders.^{38,39} PTSD, in contrast to depression, is generally considered to be associated with enhanced inhibition of the HPA axis.^{40,41} Our results demonstrate that the administration of GR- or GABA-antagonists just before stress exposure prevented enhanced inhibition of the HPA axis in rats that had undergone TBI via moderate CCI. The therapeutic potential of a GR antagonist in PTSD has been suggested based on evidence for its prevention of behavioral changes in a rodent model of PTSD.⁴² Whether there is any therapeutic potential for GR- and GABA-antagonists in TBI associated PTSD remains to be determined.

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Author Disclosure Statement

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