Adolescent Traumatic Brain Injury Induces Chronic Mesolimbic Neuroinflammation with Concurrent Enhancement in the Rewarding Effects of Cocaine in Mice during Adulthood

Steven F. Merkel,^{1,2} Roshanak Razmpour,¹ Evan M. Lutton,¹ Christopher S. Tallarida,^{2,4}
Nathan A. Heldt,¹ Lee Anne Cannella,¹ Yuri Persidsky,^{1,2}
Scott M. Rawls,^{2,4} and Servio H. Ramirez^{1–3}

Abstract

Clinical psychiatric disorders of depression, anxiety, and substance abuse are most prevalent after traumatic brain injury (TBI). Pre-clinical research has focused on depression and anxiety post-injury; however, virtually no data exist examining whether the preference for illicit drugs is affected by traumatic injury in the developing adolescent brain. Using the controlled cortical impact (CCI) model of TBI and the conditioned place preference (CPP) assay, we tested the underlying hypothesis that brain injury during adolescence exacerbates the rewarding properties of cocaine in adulthood possibly through an active inflammatory status in the mesolimbic pathway. Six-week old, C57BL/6 mice sustained a single CCI-TBI to the right somatosensory cortex. CPP experiments with cocaine began 2 weeks post-TBI. Animals receiving cocaine displayed significant place preference shifts compared to saline controls. Further, within the cocaine-experienced cohort, moderate CCI-TBI during adolescence significantly increased the preference shift in adulthood when compared to naïve controls. Additionally, persistent neuroinflammatory responses were observed in the cortex, nucleus accumbens (NAc), and ventral tegmental area post-CCI-TBI. Significant increases in both astrocytic, glial fibrillary acidic protein, and microglial, ionization basic acid 1, markers were observed in the NAc at the end of CPP testing. Moreover, analysis using focused array gene expression panels identified the upregulation of numerous inflammatory genes in moderate CCI-TBI animals, compared to naïve controls, both in the cortex and NAc at 2 weeks post-TBI, before onset of cocaine administration. These results suggest that sustaining moderate TBI during adolescence may augment the rewarding effects of psychostimulants in adulthood, possibly by induction of chronic mesolimbic neuroinflammation.

Keywords: addiction; adolescent; neuroinflammation; psychostimulants; TBI

Introduction

INDIVIDUALS UNDER THE AGE OF 25 sustain the majority of nonfatal traumatic brain injuries (TBIs).¹ These injuries typically produce short-term disruptions in consciousness and minimal tissue damage that may fall below the detection of current screening modalities.^{1,2} Even in the absence of identifiable lesions, persistent symptoms are often observed in the field of clinical psychiatry.³ Previous studies have shown that TBI is a significant predictor of an increase in the number of psychiatric diagnosis post-injury.⁴ Depression, anxiety, and substance use disorders are the most common psychiatric diagnoses among TBI patients.³ Pre-clinical research has largely focused on the assessment of depression and anxiety post-injury using the forced swim test and elevated

plus maze assays; however, few reports have examined whether adolescent TBI affects performance in behavioral models of substance abuse.

The results of a recent clinical study by Corrigan and colleagues suggest that the younger the age of first TBI, the greater the effect on problematic alcohol consumption and use of illicit drugs later in life.⁵ These findings are based upon patient responses to the Ohio State University Traumatic Brain Injury Identification (OSU-TBI-ID) survey. This survey is designed to obtain the lifetime history of brain injury from current TBI patients. Responses to the Corrigan and colleagues OSU-TBI-ID surveys reveal that approximately 8% of their cohort (moderate/severe TBI patients referred for rehabilitation services) sustained their first TBI under 16 years of age.⁵ Similarly, using the OSU-TBI-ID, Olson-Madden and colleagues

¹Department of Pathology and Laboratory Medicine, the ²Center for Substance Abuse Research, the ³Shriners Hospitals Pediatric Research Center, and the ⁴Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania.

report that approximately 54% of their cohort experienced their first TBI before adulthood.⁴ Notably, the Olson-Madden and colleagues cohort consists of military veterans seeking outpatient treatment for substance abuse. Further, a study by Ramesh and colleagues suggests that a history of TBI may be an important risk factor for the onset of cocaine use. This inference is derived from finding that 84% of their cocaine-dependent research volunteers with a history of TBI sustained their first brain injury (mean age, 16.32 ± 7.87 years) before the initiation of cocaine use (mean age, 22.97 ± 6.06 years).⁶

In addition to persistent psychiatric sequelae that can arise post-injury, the occurrence of chronic neuroinflammation has been well characterized in TBI. Autopsy evidence from moderate/severe TBI patients reveals activated-amoeboid microglia in the white matter from 2 weeks to 18 years post-injury.⁷ This morphological change is accompanied by increased expression of numerous inflammatory proteins, including the microglial marker, ionized calcium binding adaptor molecule 1 (IBA-1), which helps to regulate microglial physiology.⁸ Moreover, astrocytes contribute to chronic neuroinflammation post-TBI. The astrocytic reaction can be observed by increased expression of glial fibrillary acidic protein (GFAP) and often parallels the upregulation of IBA-1.⁸ This coordinated response suggests that astrocytes and microglia adapt post-TBI, resulting in a persistent neuroinflammatory signature. Notably, pre-clinical research has identified enhanced GFAP immunoreactivity not only at the site of injury and within the white matter, but also in subcortical regions implicated in drug addiction, such as the nucleus accumbens (NAc) and ventral tegmental area (VTA).^{9–12}

Therefore, we have combined two experimental animal models: the conditioned place preference (CPP) assay (an indicator of drug reward) and controlled cortical impact (CCI), to test the hypothesis that brain injury during adolescence exacerbates addiction-like behavior toward an illicit drug of abuse in adulthood. Our data point to a strong connection between moderate TBI and enhanced preference for cocaine, a psychostimulant that produces consistent behavioral effects in the CPP assay.^{13,14} The following studies were designed to provide important insight regarding the effect of adolescent TBI on mesolimbic neuroinflammation and susceptibility to illegal drug use during adulthood; further, our study's innovative approach could decipher underlying mechanisms of causality and targets for intervention in substance use disorders.

Methods

Animals

The Institutional Animal Care and Use Committee at Temple University (Philadelphia, PA) approved all procedures detailed in this section that required the use of vertebrate animals before initiating any experimental objectives. Six-week old, male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the University Laboratory Animal Research (ULAR) facilities at Temple University School of Medicine, Medical Education and Research Building (Philadelphia, PA) on a 12-h light-dark cycle. Upon arrival, animals were group housed for the first 48 h in order to acclimate the mice to the ULAR facility. After the acclimation period, animals were separated and housed in single cages for 24 h before the induction of experimental TBI. Throughout the entire course of housing in the ULAR facilities, animals were allowed free, unlimited access to standard calorie chow and fresh water.

Experimental traumatic brain injury using controlled cortical impact

Animals were weighted 24 h before surgery to obtain a baseline for monitoring the health status of animals after CCI procedures.

On the day of surgery, animals were weighed and anesthetized using a solution of ketamine/xylazine (Henry Schein Animal Health, Dublin, OH) administered through intraperitoneal (i.p.) injection at a dose of 100/10 mg/kg. Depth of anesthesia was monitored throughout the surgical procedure by hindpaw toe pinches to assure that animals remained properly sedated. Once anesthetized, animals were shaved to remove hair from the scalp surrounding the surgical area using a cordless trimmer (Harvard Apparatus, Holliston, MA). Animals were immobilized using a Just for MouseTM Stereotaxic Instrument (Stoelting Co., Wood Dale, IL). Ophthalmic ointment (Dechra Veterinary Products, Overland Park, KS) was applied to the eyes of immobilized animals to prevent ocular drying. Seventy percent isopropyl alcohol was used to wash the scalp, neck, and ears of surgical animals. A Zeiss Stemi 2000-C stereomicroscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) equipped with a SCHOTT EasyLED Ringlight (SCHOTT North America Inc., Elmsford, NY) was used to magnify and illuminate the surgical site. Surgical instruments were autoclaved before use and sterilized between animals using a Hot Bead Sterilizer (Fine Science Tools Inc., Foster City, CA). Surgical scissors were used to remove a portion of the scalp and expose the skull from the sagittal suture to the right temporalis muscle. The underlying fascia was removed, and an Ideal Micro-DrillTM (CellPoint Scientific Inc., Gaithersburg, MD) with a 0.5-mm, rounded burr was used to create a 4-mm craniotomy between bregma and lambda. The surgical area was periodically washed with 1× phosphate buffered saline (PBS; Corning Inc., Manassas, VA), and drill time was minimized in order to avoid overheating of the surgical area. The bone fragment resulting from the craniotomy surgery was carefully lifted away from the surgical site to avoid disrupting the underlying dura and cortical tissue. An Impact OneTM Stereotaxic CCI Instrument (Leica Microsystems, Buffalo Grove, IL) outfitted with a piston (2-mm diameter) was secured to the stereotaxic stage and positioned over the craniotomy. The piston was oriented parallel to the cortical plane and lowered until contact was made with the dural surface. Two degrees of neurotrauma were tested in the subsequent procedures by adjusting impact velocity and depth along the following parameters: velocity, 2.0 m/s; depth, 1 mm (mild); or velocity, 4.5 m/s; depth, 2 mm (moderate). A dwell time constant of 500 ms was maintained for both degrees of neurotrauma. After discharging the impactor piston, the site of injury was covered by a sterile, 5-mm glass cover-slip (Electron Microscopy Sciences, Hatfield, PA), which was secured to the skull using VetbondTM tissue adhesive (3M, St. Paul, MN), creating a waterproof seal between the glass cover-slip and the surgical margins of the scalp. This cranial window served as means to monitor the injury site visually after the craniotomy surgery. Further, this was done in order to evaluate whether experimental animals sustained additional injury (i.e., meningeal bleeding) as a result of animal handling during i.p. injection procedures in the CPP assay. Any animal with abnormal gross tissue morphology visualized through the cranial window was excluded from the study. After adhering the cover-slip atop the craniotomy, animals were removed from the stereotaxic stage and placed in their home cage, resting upon an isothermal pad (Braintree Scientific, Inc., Braintree, MA) to maintain body temperature during recovery. Animals were monitored in their home cage until consciousness was regained. After surgery, animals were weighed and surgical margins were inspected daily for 7 days. Cranial windows were also inspected on each day of behavioral testing. Animals displaying physical distress or greater than a 10% reduction in body weight were immediately euthanized. Surgical control (craniotomy only) procedures included all of the steps described above minus impactor discharge. Nonsurgical (naive) control animals were housed in single cages at the same time as experimental animals; however, these mice did not receive an i.p. injection of ketamine/xylazine and were not weighed daily for 7 days.

Locomotor activity monitoring

One week after CCI surgery, animals from all experimental groups were evaluated for locomotor deficits. Animals acclimated to the behavioral testing room in the ULAR facilities in their home cages for 30 min before initiating the locomotor activity assay. Animals were placed into a clean, translucent open-field chamber (approximately 18" L × 14" W × 8" H) for a 30-min testing session. Activity in the open field was monitored using the AccuScan Home Cage Activity System (Omnitech Electronics, Inc., Columbus, OH), which consists of a cage frame that houses a set of 16 photobeams arranged along the horizontal axis of the testing chamber, and a pair of sensor panels used to detect the beams. Fusion Software (Omnitech Electronics, Inc.) was used to collect data from the sensor panels across multiple home cage system variables in 5-min intervals over the entire 30-min testing session. Locomotor activity has been defined as the combination of ambulatory activity counts and stereotypy activity counts recorded as part of the home cage system variables. Ambulatory activity counts are the number of photobeam interruptions detected by the sensor panel as ambulation by an animal occurs in the open field. Stereotypy activity counts are the number of photobeam breaks that occur repeatedly at the same set of photobeams, which accounts for all nonambulatory activity in the open field, but does not distinguish between individual stereotypies, such as grooming or head bobbing behaviors. Together, these variables divulge information pertaining to the total activity of an animal. Any surgical mice falling below 1.5 times the standard deviation from the mean total activity counts of no craniotomy control animals were considered impaired and excluded from the CPP assay. At the end of the testing session, animals were removed from the open-field chamber and placed into their home cages and returned to an animal housing room in the ULAR facilities.

Conditioned place preference assay

A three-phase, biased CPP assay was used to assess preference for a drug-paired environment. The CPP assay was performed using a custom-designed Plexiglas chamber (approximately 13.75" L × 5.25" W × 5.00" H) manufactured by Shore Plastics (Philadelphia, PA). A removable divider wall was used to separate the chamber into two, equal-sized compartments. To establish diverse environments in the CPP chamber, each compartment was designed to be both visually and texturally distinct. One compartment had black walls and a grit-textured floor. The other compartment had white and black striped walls and a smooth-textured floor. The first phase consisted of a 30-min pre-test, which was used to establish compartment bias. During the pre-test, an opening in the compartment divider wall allowed animals to move freely between the two compartments. Animals were monitored for residence time in each compartment of the CPP chamber for 30 min. The compartment that an animal resided in for more than 15 min (> 50% of the total test time) was designated as the preferred compartment. The alternate (nonpreferred) compartment was assigned as the drug-paired compartment for the second phase of the CPP assay. All pre-tests were performed on day 15 post-CCI injury, followed by the second (conditioning) phase of testing, which consisted of 6 days of noncontingent cocaine administration on days 16–21 post-injury. On each of the 6 conditioning days, animals received an i.p. injection of cocaine (dosage, 10 mg/kg) dissolved in 0.9% sterile saline in the morning, whereas a separate group of control animals received an equal volume of 0.9% sterile saline without drug. All animals were immediately confined to their drug-paired (nonpreferred) compartment for 30 min. During the conditioning phase of the CPP assay, the door opening in the compartment divider was replaced by a solid wall to prevent animals from residing in the alternate (pre-test: preferred) compartment. In the afternoon of each conditioning day, 4 h after cocaine exposure,

all experimental animals received an i.p. injection of 0.9% sterile saline (at volumes equivalent to the morning injections) followed immediately by confinement in their preferred (drugless) compartment for 30 min. Animals were weighed in the morning on the first, third, and sixth day of cocaine conditioning in order to deliver a correct, intended dose of cocaine throughout the conditioning phase. On day 22 post-injury, animals entered the third phase of CPP testing, which consisted of a 30-min post-test. During the post-test, again, an opening in the compartment divider wall allowed animals to move freely between the two compartments. Animals were monitored for residence time in each compartment of the CPP chamber for 30 min. Place preference shifts were calculated by subtracting the residence time in the nonpreferred compartment during the pre-test from the residence time in the nonpreferred (drug-paired) compartment during the post-test. In order to increase precision and control for behavior not provoked by cocaine conditioning, only animals producing shift values between 90 and 500 sec were included in the statistical analysis. At the end of all testing sessions, animals were removed from the CPP chamber and returned to their home cages in an animal housing room of the ULAR facilities.

Tissue preparation and histology

After the CPP assay, brain tissue was harvested from all experimental animals on day 29 post-CCI. Animals were anesthetized using 4% Isoflurane and perfused transcardially, first with 20 mL of 1× PBS (Corning Inc.) followed by 20 mL of Poly/LEM Fixative (Polysciences, Inc., Warrington, PA). After perfusion, brains were extracted from the skull and immediately post-fixed in Poly/LEM Fixative for 24 h at 4°C then dissected into 2-mm-thick segments using an Alto stainless steel 1-mm brain matrix oriented in the coronal plane (CellPoint Scientific Inc., Gaithersburg, MD). Segments were post-fixed in Poly/LEM Fixative for another 24 h at 4°C. After the second 24-h round of post-fixation, brain segments were washed three times with 1× PBS and processed using a Tissue-Tek® VIP® 6 (Sakura Finetek USA, Inc., Torrance CA). The tissue was then paraffin-embedded using a TN-1500 Embedding Console System (Tanner Scientific, Inc., Sarasota, FL) and serial sectioned at 5 µm on charged slides using a rotary microtome (Leica Microsystems). Sections were heated to 56°C for 60 min before immunofluorescence staining.

Immunofluorescence assay

Serial sections of paraffin-embedded tissue were cleared and rehydrated for antigen retrieval. Sections were incubated at 95°C for 30 min in pre-warmed citrate buffer (10 mM of citric acid in distilled water, 0.05% Tween 20, pH 6.0). After antigen retrieval, sections were cooled to room temperature and rinsed with distilled water. Sections were then incubated with Background Punisher (Biocare Medical, LLC, Concord, CA) at room temperature for 30 min before application of primary antibodies. Sections were incubated in a humidifying chamber overnight at 4°C with primary antibodies: mouse antimouse GFAP (Cell Signaling Technology, Inc., Danvers, MA) and rabbit antimouse IBA-1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) diluted in 2% normal donkey serum (Jackson ImmunoResearch Inc., West Grove, PA) at ratios of 1:500 and 1:200, respectively. After overnight incubation, sections were warmed to room temperature for 1 h, then rinsed with 1× PBS (Corning Inc.) three times before application of secondary antibodies: donkey antimouse Alexa Fluor 488 (Thermo Fisher Scientific, Inc., Waltham, MA) and donkey anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific). Secondary antibodies were diluted in 2% normal donkey serum both at a ratio of 1:500. Sections were incubated with secondary antibodies in a dark humidifying chamber for 1 h at room temperature. After incubation with secondary antibodies,

sections were rinsed with 1×PBS three times, then counterstained using ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) mounted with coverslips, and stored at 4°C before immunofluorescence microscopy.

Immunofluorescence microscopy and image analysis

Fluorescent images were acquired using a Coolsnap EZ CCD camera (Photometrics, Tucson, AZ) connected to an Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY) with a solid-state Lumencore SOLA light engine[®]. Equal capture parameters were used to acquire fluorescent images from all subjects. AxioVision (Carl Zeiss Microscopy) was also used to pseudocolor the captured images. GFAP and IBA-1 positivity were quantified based upon area and fluorescent intensity using ImageJ software (National Institutes of Health, Bethesda, MD). A binary image was processed in ImageJ after defining a threshold for fluorescence intensity. Particle counting (GFAP or IBA-1) based on area was then used to determine the final number per area under observation (ImageJ software).

Real-time polymerase chain reaction

Brains were harvested from CCI-TBI (moderate) and no craniotomy control animals at 24 h and 2 weeks post-CCI surgery. Animals were transcardially perfused with 1× PBS (Corning), after which brains were removed and segmented using a mouse brain matrix oriented in the coronal plane (CellPoint Scientific Inc., Gaithersburg, MD). Segments containing the NAc and site of impact were placed immediately into RNAlater[®] Solution (Thermo Fisher Scientific) and stored at 4°C overnight before being transferred to −20°C for long-term storage. The ipsilateral region of the NAc and cortical tissue from the site of impact were excised using a brain microdissection tool 1.25 mm in diameter (Stoelting Co., Wood Dale, IL). For each N, the ipsilateral region of the NAc and cortical tissue from the site of impact from 2 experimental animals were pooled before proceeding with RNA isolation. Total RNA was isolated from the regions of interest using TRIzol[®] Reagent (Thermo Fisher Scientific) and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). A High Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific) and an Eppendorf Mastercycler[®] pro (Eppendorf AG, Hauppauge, NY) were used to prepare the complementary DNA (cDNA) for real time polymerase chain reaction (PCR). The prepared cDNA was then mixed with nuclease-free water and TaqMan[®] Fast Universal PCR Master Mix (Thermo Fisher Scientific) and added to Applied Biosystems[®] TaqMan[®] Array Mouse Immune Response 96-well Fast Plates (Thermo Fisher Scientific) containing probes for immune response-associated genes. Data were analyzed using ExpressionSuite Software (Thermo Fisher Scientific) using the delta-delta threshold cycle (Ct) method (Relative Quantification). Data are expressed as the relative fold change compared to naïve controls.

Statistical analysis

Data were analyzed for statistical significance using Prism software (version 6; GraphPad Software Inc., La Jolla, CA). Student's *t*-tests and two-way analysis of variance (ANOVA) with Tukey's post-hoc tests were performed to analyze the place preference shift data from our cocaine CPP assay. Cumulative total activity counts and GFAP and IBA-1 positivity in the NAc and VTA were analyzed by one-way ANOVA followed by Tukey's post-hoc tests. Our mouse immune response panel was analyzed by two-way ANOVA with Sidak post-hoc tests. For all tests, statistical significance was defined at $p \leq 0.05$.

Results

Controlled cortical impact induces acute neuroinflammation in the cortex and nucleus accumbens

It is well accepted that TBI unleashes a temporal cascade of inflammatory mediators that affect the neurovascular unit.^{15–17} Unfortunately, what those mediators are and where they are present is not well defined, particular in regard to subcortical structures where the reward pathway is found. Therefore, we first analyzed the acute neuroinflammatory profile post-CCI-TBI at the site of injury and at the NAc, a key input area in the reward circuitry. Whole-brain tissue was harvested 24 h post-CCI-TBI from animals that were grouped by degree of injury: naïve, craniotomy only (sham), CCI-TBI (mild), or CCI-TBI (moderate). Immunofluorescence labeling was used to determine the activation status of astrocytes and microglia in the somatosensory cortex and NAc (ipsilateral to impact). Increases in GFAP and IBA-1 immunoreactivity reflect the activation of astrocytes and microglia, respectively. As expected, basal level expression of GFAP and IBA-1 was observed in naïve and craniotomy-only controls both in the cortex and NAc (Figs. 1A,B and 2A,B). CCI-TBI (mild) animals exhibited an enhancement in astroglial GFAP expression in the cortex, mainly in the meninges and around parenchymal blood vessels (Fig. 1C), whereas only a slight increase in immunoreactivity was observed in the NAc (Fig. 2C). Similarly (Figs. 1C and 2C), upregulation of IBA-1 indicated microglial activation in the two areas under observation. CCI-TBI (moderate) induced pronounced activation of astrocytes (evident by high expression of GFAP) visible at the cortical impact site both throughout the parenchymal tissue and at surrounding blood vessels (Figs. 1D and 2D). In the NAc, IBA-1 immunoreactive staining was morphologically consistent with the presence of reactive microglia (Figs. 1D and 2D). Taken together, these analyses show that astrocytic and microglial activation is evident not only acutely at the site of impact post CCI-TBI, but also in a critically important region of the reward pathway (i.e., the NAc).

Based upon the robust glial activation observed in both the cortex and the NAc of CCI-TBI (moderate) animals, studies were conducted to explore whether inflammatory signatures would be different between the area directly affected by the impact (cortex) and the more distal NAc. To this end, using a focused inflammatory gene array panel, precise tissue “punch-outs” from the cortex and the NAc were obtained (see Methods section) to compare the inflammatory gene expression profile of these two regions. The TaqMan[®] array from Applied Biosystems (Foster City, CA) is designed to evaluate 92 genes associated with the mouse immune response along with four candidate endogenous control genes. DNA probes in the array target genes that enable immune system physiology. These genes fall into the following subcategories: cytokine receptors, cytokines, chemokines, chemokine receptors, transcription factors, cell surface receptors, stress response, oxidoreductases, protein Kinases, cell cycle, signal transduction, and proteases.

After RNA extraction and cDNA conversion, the results of the assay revealed that 51 genes were upregulated, eight genes were downregulated, and 35 genes showed no change in the cortex after the acute phase of injury (Fig. 1E,G). These analyses are shown as the fold regulation of CCI-TBI (moderate) over naïve control. Significant change was defined at ± 1.5 fold. Genes upregulated between 1.5 and ≤ 10 included: CCR2, CCR4, CCR7, CD40LG, CD68, CD80, CD86, CD8a, CSF1, CXCL11, CYP1a2, Edn1, Fas,

FIG. 1. Neuroinflammation in the cortex 24h post-CCI-TBI. Representative images from the somatosensory cortex (ipsilateral to impact) at 20× objective magnification with individual left insert images: GFAP (white), IBA-1 (magenta), DAPI (cyan), and a composite large merged image to the right (scale bars=50 microns). (A) Naive control, (B) craniotomy-only control, (C) CCI-TBI (mild), and (D) CCI-TBI (moderate). A real-time quantitative polymerase chain reaction (PCR) focused array panel for inflammatory genes was used to profile the inflammatory response in animals that had sustained a CCI-TBI in comparison to those without. Total RNA was extracted from the cortex (ipsilateral to the impact), reverse-transcribed, and the corresponding complementary DNA was evaluated by real-time PCR as described in the Methods section. (E) Gene regulation results in graph form for genes beginning with letters A–H. (F) Gene regulation results in graph form for genes starting with letters I–V. (G) Average fold regulation values shown along with their target genes derived from graphs E and F; red labeled values denote significant upregulation (with a threshold set at 1.5-fold), whereas blue indicates significant downregulation (with a threshold set at –1.5-fold). Expression profiles were analyzed using the $\Delta\Delta C_t$ method, and the results are represented as the average fold change in gene expression of CCI-TBI over naïve control $\pm$ standard error of the mean; asterisks indicate statistical significance defined as $p \leq 0.05$. CCI, controlled cortical impact; $\Delta\Delta C_t$, delta-delta threshold cycle; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA-1, ionization basic acid 1; TBI, traumatic brain injury.

FIG. 2. Neuroinflammation in the nucleus accumbens (NAc) 24 h post-CCI-TBI. Representative images from the NAc (ipsilateral to impact) at 20 $\times$ objective magnification with individual left insert images: GFAP (white), IBA-1 (magenta), DAPI (cyan), and a composite large merged image to the right (scale bars=50 microns). (A) Naïve control, (B) craniotomy-only control, (C) CCI-TBI (mild), and (D) CCI-TBI (moderate). A real-time quantitative polymerase chain reaction (PCR) focused array panel for inflammatory genes was used to profile the inflammatory response in animals that had sustained a CCI-TBI in comparison to those without. Total RNA was extracted from the NAc (ipsilateral to the impact), reverse-transcribed, and the corresponding complementary DNA was evaluated by real-time PCR as described in the Methods section. (E) Gene regulation results in graph form for genes beginning with letters A–H. (F) Gene regulation results in graph form for genes starting with letters I–V. (G) Average fold regulation values shown along with their target genes derived from graphs E and F; red labeled values denote significant upregulation (with a threshold set at 1.5-fold), whereas blue indicates significant downregulation (with a threshold set at -1.5-fold). Expression profiles were analyzed using the $\Delta\Delta C_t$ method, and the results are represented as the average fold change in gene expression of CCI-TBI over naïve control $\pm$ standard error of the mean; asterisks indicate statistical significance defined as $p \leq 0.05$. CCI, controlled cortical impact; $\Delta\Delta C_t$, delta-delta threshold cycle; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA-1, ionization basic acid 1; TBI, traumatic brain injury.

FasL, H2-Ea, H2Eb1, ICAM-1, IL13, IL17a, IL1 α , IL2, IL7, Ly96, NF κ B1, NF κ B2, Nos2, Ptg2, Ptpcr, Selp, Smad3, Soc1, Stat1, Stat3, TGF β 1, and TNF α . Genes upregulated between 10 and ≤ 100 included: C3, CCL5, CSF2, CSF3, Hmox1, IL12b, IL9, and Sele. Genes upregulated ≥ 100 included: CCL2, CCL3, CXCL10, IL10, IL1 β , IL6, and Lif. The genes that were downregulated (≤ -1.5) included: CD28, CD3e, Cyp7a1, IL12a, IL15, IL5, Lta, and NFATc4. Of note, the fold regulation of factors, such as CCL2/MCP-1, CXCL10/IP10, IL1 β , and IL6, which promote neuroinflammation, is highly elevated.

Analysis of the inflammatory profile for the NAc showed a markedly different result (Fig. 2E,G). The number of upregulated genes was 17. Compared to the cortex, gene upregulation in the NAc was modest (no genes above 100 fold). Genes upregulated between 1.5 and ≤ 10 included: CCL2, CCL3, CD28, CYP7a1, Hmox1, ICAM-1, IL17a, IL1 α , Lif, NF κ B2, Ptg2, Sele, Stat3, and Tbx21. Genes upregulated between 10 and ≤ 100 included: CSF3, IL1 β , Selp, and TNF α . Interestingly, an equal number of genes (17) were downregulated. Genes that were downregulated below -1.5 included: CCL5, CCR7, CD19, CD40LG, CSF2, CXCL11, CXCR3, CYP1a2, H2-Eb1, IL12a, IL12b, IL13, IL2, IL4, Lrp2, and Prf1. These results are consistent with the activation of astrocytes and microglia (as observed by immunofluorescence) and

provide a more comprehensive view of the inflammatory signature that parallels the enhanced immunoreactivity of GFAP and IBA-1 post-CCI-TBI (moderate).

Place preference shifts are significantly enhanced in animals sustaining moderate controlled cortical impact traumatic brain injury

The following studies were designed to test the underlying hypothesis that TBI may alter the reward circuitry in the developing brain of adolescent mice, which subsequently could lead to an increase in addiction-like behavior. We delayed behavioral testing by 1 week to allow for resolution of the primary injury. A schematic depicting the time at which CCI-TBI was induced in relation to the behavioral assays and tissue harvesting is shown in Figure 3A. At 2 weeks post-CCI-TBI, a biased CPP assay was run to assess drug reward and reinforcement in our experimental animals. Cocaine was administered once-daily at a dose of 10 mg/kg for 6 consecutive days. This dose has previously been shown to produce an optimal shift in the CPP assay.¹⁸ Control animals received an equivalent volume of 0.9% saline. Further, for experimental animals, the site of injury was monitored visually on each day of behavioral testing in order to rule out the potential for additional injury (i.e., meningeal bleeding) in CCI and craniotomy-only mice, which might occur as a result of animal handling during administration of cocaine. To this end, 4 CCI-TBI (moderate), 0 CCI-TBI (mild), and 6 craniotomy-only animals were removed from the study because of additional injuries observed during post-operative care.

FIG. 3. Experimental flow schematic of endpoints and analysis of the effects of CCI-TBI on the rewarding properties of a psychostimulant. (A) Timeline of experimental endpoints. (B) Results from the CPP assay. A three-phase CPP assay was used to assess addiction-like behavior by analyzing the preference for a drug-paired environment. Animals received cocaine (10 mg/kg) dissolved in 0.9% sterile saline by intraperitoneal injection for 6 consecutive days. Control animals received equivalent volumes of 0.9% sterile saline. Place preference shift values were calculated by subtracting the residence time in the drug-paired compartment during the pre-test from the residence time in the drug-paired compartment during the post-test. Data are presented as mean $\pm$ standard error of the mean. Student's *t*-tests were performed to analyze the effect of drug (saline vs. cocaine) for each degree of injury (naive control, craniotomy only, CCI-TBI [mild], and CCI-TBI [moderate]); asterisks indicate statistical significance defined as $p \leq 0.05$. Data were further analyzed by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests for main effects of degree of injury; asterisks indicate statistical significance defined as $p \leq 0.05$. (C–F) Locomotor activity monitoring. To control for the possibility that motor impairment may affect the performance of CCI-TBI animals in the CPP assay, locomotor activity was monitored 1 week before the CPP pre-test. Animals were monitored for 30 min, which is equal to the time of all CPP sessions. Data are presented as mean $\pm$ standard error of the mean. (C and D) Ambulatory and stereotypy activity counts were recorded over 5-min intervals throughout a 30-min testing session. (E) Total activity counts (the sum of ambulatory and stereotypy activity counts) over 5-min intervals throughout a 30-min testing session. (F) Cumulative total activity counts (the sum of total activity counts over the entire 30-min testing session). Data were analyzed by one-way ANOVA followed by Tukey's post-hoc tests; NS indicates $p \geq 0.05$. CCI, controlled cortical impact; CPP, conditioned place preference; TBI, traumatic brain injury.

FIG. 4. Chronic neuroinflammation in the cortex 1 month post-CCI-TBI. Representative images from the somatosensory cortex (ipsilateral to impact) at 20× objective magnification with individual left insert images: GFAP (white), IBA-1 (magenta), DAPI (cyan), and a composite large merged image to the right. (A) Naïve control, (B) craniotomy-only control, (C) CCI-TBI (mild), and (D) CCI-TBI (moderate). Arrows point to examples of activated astrocytes or microglial cells. Scale bars=50 microns. CCI, controlled cortical impact; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA-1, ionization basic acid 1; TBI, traumatic brain injury.

For each degree of injury, animals receiving cocaine displayed significant place preference shifts for the drug-paired environment compared to their respective saline control (statistical comparisons resulted in the following: cocaine vs. saline: naïve control, $p=0.0001$; craniotomy only, $p<0.0001$; CCI-TBI [mild], $p=0.0001$; CCI-TBI [moderate], $p<0.0001$; Fig. 3B). Further, within the cocaine-experienced cohort, two-way ANOVA with Tukey's post-hoc tests revealed a statistically significant effect of CCI-TBI (moderate) compared to controls (within cocaine treatment factor: naïve control vs. CCI-TBI [moderate], $p=0.0088$; Fig. 3B). This result suggests that adolescents sustaining a single moderate TBI are increasingly susceptible to cocaine reward during adulthood and supports data obtained from clinical populations.^{5,6} Moreover, although no other significant interactions were identified in the statistical analysis, it is important to note that for each increase in the degree of injury (naïve control → craniotomy only → CCI-TBI [mild], etc.), there is a trend toward escalating place preference for the cocaine-paired compartment of the CPP chamber (see Methods section).

In order to control for the possibility that locomotor impairment arising from CCI-TBI could confound the results of the CPP assay by affecting an animal's ability to explore the testing chamber, total locomotor activity was monitored 1 week before the CPP pre-test. Total locomotor activity is the sum of ambulatory and stereotypical (Fig. 3C,D) activity. All CCI-TBI animals displayed a pattern of locomotor activity similar to controls throughout the 30-min monitoring session (Fig. 3E). A one-way ANOVA with Tukey's post-hoc

tests revealed no significant difference in cumulative total activity (i.e., the sum of total activity counts over the entire 30-min test session) between control and CCI-TBI animals ($p>0.05$), indicating that any locomotor deficits in surgical mice had resolved before the CPP pre-test and therefore did not influence the results of the CPP assay (Fig. 3F).

Chronic neuroinflammation in the cortex 1 month after controlled cortical impact traumatic brain injury

Because chronic neuroinflammation is a well-known phenomenon post-TBI, the subsequent studies were conducted to determine whether ongoing neuroinflammation in the form of glial activation could be observed not only in the cortex, but also in subcortical regions of the mesolimbic pathway. Additionally, if inflammation could be identified in the reward circuit, it would be important to know whether the inflammation was related to neurotrauma alone or comorbid psychostimulant administration. To this end, whole brains were harvested from cocaine-experienced animals 1 week after the CPP post-test (day 29 post-CCI). Sections from the ipsilateral somatosensory cortex were prepared for detection of GFAP and IBA-1 by immunofluorescence microscopy. Similar to sections obtained from animals 24 h post-injury, minimal immunoreactivity for these targets could be observed in naïve and craniotomy-only controls (Fig. 4A,B), suggesting that drug administration during the CPP assay has little effect on glial immune activation in the cortex

after 1 week of cocaine abstinence. However, sections from both CCI-TBI (mild) and CCI-TBI (moderate) animals displayed prominent GFAP and IBA-1 immunopositivity (Fig. 4C,D). Further, the fluorescent intensity of these targets in injured mice appeared greater than could be detected only 24 h post-CCI (Fig. 1C,D). Comparable GFAP and IBA-1 immunoreactivity could be observed at the site of impact in saline control CCI-TBI animals (data not shown), further indicating that neurotrauma alone (regardless of cocaine exposure) precipitates a stable neuroinflammatory response in the cortex.

Moderate controlled cortical impact traumatic brain injury induces significant chronic neuroinflammation in the nucleus accumbens 1 month post-injury

The strong presence of glial activation in the cortex of CCI-TBI animals at 1 month prompted the realization that perhaps a more intense glial response would be visible in areas of the reward pathway.

Focus was once again turned to the NAc because glial activation and upregulation of proinflammatory mediators was observed in the acute phase of injury. Importantly, failure to resolve inflammation in the NAc may explain the robust place preference shift of CCI-TBI (moderate) animals in the CPP assay. Therefore, sections from the NAc (approximately 1.70 mm from the bregma) were prepared for GFAP and IBA-1 immunofluorescence microscopy. As observed in the cortex, neuroinflammatory response appeared more intense in the NAc 1 month post-injury than at 24-h. Again, minimal immunoreactivity was observed in naïve and craniotomy-only controls (Fig. 5B,C); however, distinct GFAP and IBA-1 immunoreactivity could be observed throughout the NAc of CCI-TBI (mild) and CCI-TBI (moderate) animals (Fig. 5D,E). Further, glial immune response was not only isolated to vascular areas (counterstain not shown) as observed acutely post-injury, but extended deep into the parenchyma of CCI-TBI (moderate) animals (Fig. 5A,E). Moreover, the contralateral NAc of cocaine-experienced CCI-TBI animals displayed GFAP and

FIG. 5. Chronic neuroinflammation in the nucleus accumbens (NAc) 1 month post-CCI-TBI. (A) Strategy employed to quantify glial activation post-TBI in the NAc: includes a representative image from tissue sectioned from the ipsilateral NAc of an animal that was exposed to CCI-TBI (moderate); 4× objective magnification of a composite merged image of tissue immunolabeled for GFAP (white), IBA-1 (magenta), and counterstained with DAPI (cyan). The section is approximately 1.70 mm from bregma. The anterior commissure (aca) is outlined in white dashed lines; the accumbens core is labeled (AcbC), and the entire NAc is outlined (larger perimeter) by yellow dashed lines. (B–E) Representative images from the NAc (ipsilateral to impact) of cocaine-experienced animals; 20× objective magnification with individual left insert images: GFAP (white), IBA-1 (magenta), DAPI (cyan), and a composite large merged image to the right (scale bars = 50 microns). (B) Naïve control, (C) craniotomy-only control, (D) CCI-TBI (mild), and (E) CCI-TBI (moderate). (F and G) Quantification of GFAP and IBA-1 immunoreactive cells in the NAc of cocaine-experienced animals. Data are presented as mean ± standard error of the mean. Equal acquisition parameters were used to acquire all fluorescent images for quantification. Image analysis was performed by processing the images for: area calibration, background subtraction, segmentation, threshold intensity, conversion to binary, and particle counting (based on size and circularity). The sum was calculated as a function of particle counting. Data were analyzed by one-way analysis of variance followed by Tukey's post-hoc tests; asterisks indicate statistical significance defined as $p \leq 0.05$. CCI, controlled cortical impact; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA-1, ionization basic acid 1; TBI, traumatic brain injury.

IBA-1 immunoreactivity comparable to naïve and craniotomy-only controls (data not shown), again indicating that neuroinflammation in the ipsilateral NAc was TBI dependent and not related to cocaine exposure.

To better understand the degree of glial activation, image analysis was then performed in order to quantify the number of activated astrocytes and microglia. The image analysis quantification strategy is shown in Figure 5A. Glial immune response in the NAc was quantified by acquiring multiple images from around the anterior commissure (aca), followed by isolating the corresponding channel, then processing the image for area calibration, background subtraction, segmentation, threshold intensity, conversion to binary, and particle counting (based on size and circularity). The sum

was calculated as a function of total area and averaged across animals in each group (Fig. 5A). Compared to naïve and craniotomy-only controls, both GFAP and IBA-1 immunoreactivity were significantly enhanced in the NAc of CCI-TBI (moderate) animals (one-way ANOVA of GFAP-positive cells: naïve control vs. CCI-TBI [moderate], $p=0.0003$; craniotomy-only vs. CCI-TBI (moderate), $p=0.0100$; and one-way ANOVA of IBA-1-positive cells: naïve control vs. CCI-TBI [moderate], $p=0.0032$; craniotomy-only vs. CCI-TBI [moderate], $p=0.0018$; Fig. 5F,G). Notably, this enhancement correlates with the significant increase in the CPP shift of CCI-TBI (moderate) animals, suggesting that glial immune activation in the NAc may contribute to the enhanced behavioral response to cocaine.

FIG. 5. (Continued)

Astrocyte activation in the ventral tegmental area 1 month after controlled cortical impact injury

Post-synaptic neurons in the NAc receive dopaminergic signals from cells originating in the VTA.^{19–21} This mesolimbic circuit is heavily implicated in drug abuse behavior; therefore, we also analyzed GFAP and IBA-1 expression in the VTA. As previously observed in the cortex and NAc of cocaine-experienced animals,

naïve and craniotomy-only controls displayed minimal immunoreactivity (Fig. 6A,B). Further, CCI-TBI (mild) animals only exhibited slight increases in GFAP and IBA-1 expression (Fig. 6C). However, distinct GFAP immunoreactivity could be observed in the VTA of CCI-TBI (moderate) animals, whereas IBA-1-positive cells appeared unremarkable (i.e., normal baseline intensity and displaying a resting morphology; Fig. 6D). Quantification of GFAP and IBA-1 from serial sections of the VTA is shown in Figure 6E

FIG. 6. Neuroinflammation in the ventral tegmental area (VTA) 1 month post-CCI-TBI shows astrocyte, but not microglial, activation. Representative images from the VTA (ipsilateral to impact) of cocaine-experienced animals; 20× objective magnification with individual left insert images: GFAP (white), IBA-1 (magenta), DAPI (cyan), and a composite large merged image to the right. (A) Naive control, (B) craniotomy-only control, (C) CCI-TBI (mild), and (D) CCI-TBI (moderate). Arrows point to examples of activated astrocytes. Scale bars=50 microns. (E and F) Quantification of GFAP and IBA-1 immunolabeled cells in the VTA of cocaine-experienced animals. Data are presented as mean $\pm$ standard error of the mean. Equal acquisition parameters were used to acquire all fluorescent images for quantification. Image analysis was performed by processing the images for: area calibration, background subtraction, segmentation, threshold intensity, conversion to binary, and particle counting (based on size and circularity). The sum was calculated as a function of particle counting. Data were analyzed by one-way analysis of variance followed by Tukey's post-hoc tests; asterisks indicate statistical significance defined as $p \leq 0.05$, NS indicates $p \geq 0.05$. CCI, controlled cortical impact; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA-1, ionization basic acid 1; TBI, traumatic brain injury.

and 6F. There was no significant change in IBA-1-immunopositive cells between experimental groups 1 month post-injury ($p > 0.05$; Fig. 6F); however, CCI-TBI (moderate) animals had significantly more enhanced GFAP-positive cells in the VTA compared to controls (one-way ANOVA of GFAP-positive cells: naïve control vs. CCI-TBI [moderate], $p = 0.0206$; craniotomy only vs. CCI-TBI [moderate], $p = 0.0022$; Fig. 6E). These data suggest that astrocytes alone are stably activated in the VTA after moderate CCI.

Increased transcription of inflammatory genes in the cortex and nucleus accumbens 2 weeks after controlled cortical impact injury

Because a coordinated glial response was observed in the NAc at the end of the CPP testing, it is highly likely that an inflammatory response was already present at the time CPP testing was initiated. Additionally, characterizing the inflammatory factors expressed before psychostimulant administration may reveal target genes that influenced the heightened preference for cocaine seeking observed in CCI-TBI (moderate) animals. Further, a different inflammatory signature may arise in the subacute phase post-injury compared to that in the acute phase post-CCI-TBI (moderate). To test the likelihood of the above notions, focused gene expression array panels with DNA probes for 92 genes important in modulating the mouse immune response were utilized. Tissue was harvested from experimental animals 2 weeks post-CCI-TBI (moderate). As a point of reference, the analysis was started with the cortical area ipsilateral to the impact.

Unlike the acute phase, analysis of the gene expression array at 2 weeks post-injury revealed a more pronounced inflammatory response, showing 60 genes upregulated (Fig. 7A–C) compared to 51 (Fig. 1G). The subacute panel analysis showed an increase in mid-range fold regulation (10- to 100-fold), which resulted in 22 genes upregulated compared to eight in the acute. Many of the down-regulated genes remained the same and were also close in number, eight for the acute and six for the subacute phase. Again, the analysis reflects fold regulation of CCI-TBI (moderate) over naïve control, and significant change was defined at ± 1.5 fold. Break-down of the analysis is as follows. Genes upregulated between 1.5 and ≤ 10 included: CCR2, CD28, CD38, CD40, CD40LG, CD80, CD86, CSF1, CSF2, CSF3, CTLA4, CXCL11, CYP1A2, Fas, Fn1, Hmox1, Icos, IL12b, IL15, IL1 α , IL2ra, Lrp2, LY96, NFATc4, NF κ B1, NF κ B2, Ptprc, Sele, Socs1, Stat1, Stat3, TGF β 1, and VCAM-1. Genes upregulated between 10 and ≤ 100 included: CCL3, CCL5, CCR4, CCR7, CD3e, CD68, CD8a, CXCL10, CXCR3, FasL, H2-Eb1, ICAM-1, IFN γ , IL1 β , IL2, IL4, Lif, Prf1, Selp, Tbx21, TNFrsf18, and TNF α . Genes upregulated ≥ 100 included: C3, CCL2, CD4, and IL17a. Downregulated genes (≤ -1.5) included: CD34, Cyp7a1, IL12a, Lta, IL12a, Socs2, and Vegfz.

Analysis of the NAc in the subacute phase showed markedly different results in comparison to the acute. Notably, the majority of gene targets in the panel were significantly elevated in the NAc of CCI-TBI (moderate) animals (Fig. 7D–G). Unlike in the acute phase of injury (24 h post-TBI), analysis of the subacute NAc array revealed more-significant elevations in proinflammatory gene transcription. Results of the analysis showed that 66 of the 92 genes in the array were upregulated beyond 1.5-fold, compared to 17 in the acute phase post-CCI-TBI (moderate). Importantly, most genes (61) were upregulated at the lower range (1.5- to ≤ 10 -fold) and only five at the mid-range (10- to ≤ 100 -fold). No genes in the ipsilateral NAc were regulated ≥ 100 -fold during the subacute phase. The specific gene targets identified in the analysis were as follows.

Genes upregulated between 1.5 and ≤ 10 included: Bax, Bcl2, C3, CCL9, CCL2, CCL5, CCR2, CC4, CCR7, CD28, CD38, CD3e, CD4, CD40, CD40LG, CD80, CD86, CD8a, CSF1, CTLA4, CXCL10, CXCR3, CYP7a1, FasL, Fn1, H2-Ea, H2Eb1, Hmox1, Hprt1, ICAM-1, Icos, IKKB, IL12a, IL13, IL15, IL17a, IL1 α , IL1 β , IL3, IL6, IL7, IL9, Lta, Ly96, Prf1, Ptgs2, Ptprc, Sele, Selp, Ski, Smad3, Smad7, Socs1, Socs2, Stat1, Stat3, Stat4, Stat6, TNFrsf18, and TNF α . Genes upregulated between 10 and ≤ 100 included: CSF3, IFN γ , IL2, IL5, and Tbx21. Downregulation of genes in the NAc during the subacute phase was observed in only three genes compared to 17 genes in the acute (24 h post-TBI). The specific downregulated genes ≤ -1.5 -fold included: CD19, Cyp1a2, and IL4.

Taken together, the data point to a persistent neuroinflammatory state both in the cortex and in the NAc after adolescent CCI-TBI. One important consideration is that glial activation and the immune response in the cortex may greatly affect what occurs in the NAc. Further, in clinical TBI populations, the frontal cortex is one of the most vulnerable sites to neurotrauma.^{2,22} This is another important point to consider given that aberrant communication between the pre-frontal cortex (PFC) and NAc is heavily implicated in the neurobiology of substance abuse.^{23,24}

Discussion

The results of this study have shown that CCI initiates neuroinflammation not only at the site of impact, but also in the NAc and VTA, two subcortical regions that comprise the mesolimbic dopaminergic tract. Further, the CPP assay has shown that animals sustaining a single, moderate TBI during adolescence (6 weeks old) exhibit significantly greater preference for a cocaine-paired environment compared to controls during adulthood (9 weeks old).²⁵ Notably, this augmented behavior toward cocaine is concurrent with an evolving inflammatory response in the cortex and NAc. Two methods of analysis, immunofluorescence microscopy and real-time PCR, were used to confirm the presence of an inflammatory signature in the cortex and NAc at 24 h, 2 weeks, and 29 days post-injury. Together, these results support our hypothesis that brain injury during adolescence exacerbates addiction-like behavior toward an illicit drug of abuse in adulthood and identifies neuroinflammation in the mesolimbic tract, which is a unifying circuit that mediates the euphoric and aversive properties of drugs of abuse. Therefore, we propose that glial activation and the production of immune response genes post-TBI may augment the reinforcing effects of drugs of abuse by altering homeostasis in the reward pathway.

Our result from the cocaine CPP assay is the first pre-clinical report to demonstrate the effect of TBI on the post-injury rewarding effects of illicit drugs of abuse. Previous preclinical investigations have focused on behavioral assays directed toward alcohol abuse post-TBI. These reports have produced seemingly conflicting results. Using voluntary ethanol self-administration and fluid percussion injury (FPI), Mayeux and colleagues found that adult, male Wistar rats sustaining TBI significantly increased their alcohol intake over baseline compared to controls.⁹ Lim and colleagues produced similar results to the FPI study, but only by stratifying their cohort into upper 50% and lower 50% drinkers, finding that the upper 50% drinkers in a two-bottle choice paradigm exhibited significantly greater ethanol intake compared to controls after sustaining mild blast overpressure to induce TBI.²⁶ However, without stratifying the cohort, Lim and colleagues report that mild blast overpressure does not significantly influence ethanol intake

during intermittent access nor during 2-week periods of alcohol deprivation, which has previously been shown to increase ethanol consumption in noninjured rats.²⁶ Again, using a two-bottle choice assay, Weil and colleagues found that female mice exposed to a mild, closed head impact injury consumed significantly more alcohol compared to their sex-specific controls, whereas male animals sustaining the same type of neurotrauma displayed no difference in drinking behavior compared to their sex-specific controls.²⁷ Interestingly, both studies using the two-bottle choice assay concluded that the increase in alcohol consumption exhibited by TBI animals was not influenced by a reduced sense of palate or taste, indicating that another process must be governing the escalating alcohol intake observed post-TBI.^{26,27}

Similar to our study, Weil and colleagues evaluated the effect of juvenile TBI on adulthood drinking behavior by inducing mild closed head injury in 3-week-old mice and delaying behavioral testing until approximately 9 weeks post-TBI, whereas, we have observed the effect of CCI injury in 6-week-old mice and delayed cocaine conditioning until 2 weeks post-TBI.²⁷ Using the CPP assay as an indicator of drug-evoked reward, our results have shown enhanced sensitivity to cocaine in CCI-TBI (moderate) animals 2 weeks post-injury. Notably, Weil and colleagues also evaluated the behavioral response to alcohol reward in juvenile TBI animals using CPP. Their CPP results were consistent with those from their two-bottle choice assay, finding that only female mice injured at 21 days of age displayed a significant response to alcohol compared to sex-specific controls, whereas injured males had a similar response to controls.²⁷ Although our study has revealed an enhanced preference for the cocaine-paired environment in injured males, both our data and that of Weil and colleagues affirm the hypothesis that adolescent/juvenile TBI augments addiction-like behavior by altering the response to reward.

The sex difference that exists between our CPP results and those of Weil and colleagues may be related either to the use of cocaine or the strain of mouse used for our behavioral characterization. Psychostimulants like cocaine have been shown to produce robust CPP in C57BL/6 mice compared to ethanol.¹⁸ Therefore, we may observe a heightened CPP response in our injured male subjects simply attributable to the use of cocaine versus alcohol. Second, cocaine has a defined pharmacological target within the central nervous system (CNS), namely, the dopamine transporter (DAT), whereas alcohol lacks this specificity.^{28,29} Importantly, DAT is expressed at the synaptic terminals of dopaminergic neurons, which are bundled into four principle tracts in the CNS.²⁹ The mesolimbic pathway is one of these principle dopaminergic tracts that functions in the process of behavioral reward.^{30,31} Dopaminergic neurons of the mesolimbic pathway originate in the VTA and synapse onto medium spiny neurons in the NAc.³² By employing cocaine CPP in our study, we have not only observed augmented behavior by TBI animals in a well-validated assay of drug reward, but we have also used an illicit drug of abuse that deregulates synaptic transmission in the mesolimbic pathway by inhibiting DAT, adding credence to the hypothesis that TBI escalates addiction-like behavior by altering function in the reward pathway.

Our histology and quantitative PCR data identify the presence of an inflammatory response in the CNS at 24 hours, 2 weeks, and 29 days post-TBI not only in the cortex (at the site of impact), but also in the NAc (a component of the ventral striatum) and, to a lesser extent, in the VTA of our CCI-TBI (moderate) animals. Notably, clinical studies in neuroradiology have described white matter pathology in the NAc of TBI patients. Shah and colleagues observed reduced volume in the left NAc of severe TBI patients 6

months post-injury along with bilateral reductions in fractional anisotropy (FA) throughout the ventral striatum.³³ Further, Shah and colleagues report that FA reductions in the right ventral striatum correlated with impaired executive function (a trait commonly observed among drug abusers).^{30,33} Alhilali and colleagues also observed reduced FA in the NAc of mild TBI patients with neuropsychiatric symptoms, finding that poor white matter integrity in the NAc correlated with longer recovery time post-injury.³⁴

In addition to white matter injury, other pre-clinical studies have identified neuropathology in the mesolimbic nuclei of their TBI subjects. Consistent with our data, Sajja and colleagues noted elevated expression of Bcl-2, Bax, Caspase-3, and GFAP at 24 and 48 h after blast-induced neurotrauma, indicating early neuroinflammation and neurodegeneration in the NAc post-injury.¹¹ Using a closed head impact injury model of TBI, Lowing and colleagues observed increased GFAP expression in the NAc up to 7 days post-injury that resolved to the level of controls subjects by 2 weeks.¹⁰ We have presented data identifying increased GFAP expression in the astrocytes of the NAc up to 4 weeks post-CCI-TBI. The disparity between our results and those of Lowing and colleagues may be related to the different models of TBI (closed-head vs. cortical impact) used in these studies, given that Shin and colleagues also observed an increase in GFAP expression in the VTA 4 weeks post-injury using the CCI model, which is consistent with our VTA data.¹² Finally, Weil and colleagues observed greater c-Fos expression throughout the NAc of injured female mice approximately 90 days post-injury, revealing a persistent change in the transcriptional program of this nucleus.²⁷ Together with our data, these reports suggest that neuroinflammation and neurodegeneration occur with a concurrent loss of volume and diminished white matter integrity in the NAc of TBI subjects/patients that can affect executive function and produce neuropsychiatric symptoms.

The neurobiology of drug addiction is believed to arise partially in the PFC as a result of loss of control over executive function.^{20,24} Acute drug administration increases the concentration of dopamine in the NAc and PFC.^{19,20} Glutamatergic neurons in the PFC extend their axonal projects to the NAc, creating a corticostriatal circuit that mediates drug relapse by destabilizing glutamate homeostasis and altering synaptic morphology in the NAc, leading to a loss of executive control over reward-based behavior.²³ Chronic drug use ultimately depresses dopaminergic transmission in the NAc through induction of the dynorphin-kappa opioid system.²⁰ Briefly, dynorphin is expressed by neurons in the NAc that project back to the VTA where they release dynorphin, which activates kappa opioid receptors on dopaminergic neurons in the VTA and impedes the release of dopamine, creating the aversion and negative affect associated with drug withdrawal.^{20,31,35} Interestingly, TBI has been shown to produce similar changes in glutamate and dopamine neurotransmission and induce expression of dynorphin.^{36–38} Hinzman and colleagues recorded significant elevations in synaptic glutamate spillover in the rat striatum 2 days post-FPI.³⁹ Clinical imaging studies have observed impaired dopaminergic transmission in TBI patients, which has been corroborated by multiple preclinical reports.^{37,40–43} In fact, Wagner and colleagues specifically identified depressed dopaminergic transmission in the rat striatum 2 weeks post-TBI.³⁷ Finally, a couple publications have reported increased dynorphin expression post-TBI, with a study by Hussain and colleagues defining a lateralized response where only a right-sided TBI led to increased dynorphin expression in the striatum and frontal cortex 7 days post-CCI injury.^{38,44} Therefore, TBI may neurochemically prime the brain in a manner akin to chronic drug abuse, creating a state where those with a history of

TBI are increasingly susceptible to drug reinforcement attributed to a dysfunctional corticostriatal circuit.

Despite understanding these neurobehavioral processes in substance abuse, investigations targeting dopamine, glutamate, and the kappa opioid system have not produced any pharmacotherapies for the treatment of drug addiction. However, studies in the field of neuroimmunology have produced data to support the hypothesis that inflammatory factors mediate neuronal physiology and drive drug-evoked behavior. Multiple reports have described an interaction that occurs between opioid compounds and the innate immune receptor, Toll-like receptor 4 (TLR-4), leading to the production of interleukin (IL)-1 β , which we observed upregulated in the cortex of our CCI-TBI mice.^{45,46} Moreover, Hutchinson and colleagues have shown that TLR-4 knockout (KO) mice fail to develop opioid CPP, and Blednov and colleagues found that CD14 KO mice exhibited no increase in voluntary ethanol consumption after lipopolysaccharide (TLR-4 agonist) treatment, whereas wild-type mice significantly increased their alcohol intake.^{46,47} These studies suggest that activation of innate immunity is required to reinforce drug reward and escalate drug-taking behavior. Further, chemokines like CCL5 (which our inflammatory panel determined was overexpressed in the NAc of TBI animals) have been shown to diminish the activity of all three opioid receptors in the presence of their respective ligands though the process of heterologous desensitization, whereby activation of one G-protein-coupled receptor (GPCR) decreases the potency of ligands for alternative GPCRs.⁴⁸ This activity has lead Adler and Rogers to propose the hypothesis that chemokines act as a third major signaling system in the brain (the first two systems being the neurotransmitter and neuroendocrine systems).⁴⁸ Last, the chemokine system appears to influence the differentiation of neural progenitor cells after ischemic injury in the striatum by enhancing GABAergic innervation of these cells in the subventricular zone (a physiological trait common to newly developing neurons).⁴⁹ Moreover, Mithal and colleagues has demonstrated how chemotactic guidance in the CNS regulates cell morphology and cell fate during embryonic development.⁵⁰ Therefore, in order to discover new therapeutic targets, the activity of inflammatory factors differentially expressed in the cortex and NAc of our CCI-TBI animals may be tested to assess how their activity influences behavior in assays of drug reinforcement.

Our future investigations will be aimed at verifying the enhanced response to cocaine post-TBI in operant self-administration assays, and using a repeated closed-head injury model of TBI to discover whether there is a minimum threshold for the number of mild injuries needed to augment drug-taking behavior. We will also extend our behavioral testing to include other drugs of abuse. For instance, some current reports have noted the therapeutic potential of low-dose administration of methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) post-TBI; therefore, characterizing liability to these drugs of abuse post-TBI is of paramount importance to safeguard patient health.^{51–53} Testing prescription opiates is also important as these medicines may be offered to TBI patients for pain (especially in situations of polytrauma). In addition, expanding the number of inflammatory genes beyond those analyzed here would provide greater insight into the inflammatory signature of TBI in the NAc. In fact, future studies will provide the missing inflammatory maps for the other important areas of the brain implicated in the neurobiology of drug addiction, such as the PFC, hypothalamus, and amygdala.

In conclusion, our behavioral data show that a history of TBI in adolescent mice increases the susceptibility to psychostimulant drug reinforcement; specifically, TBI-affected animals display

greater preference for a cocaine-paired environment over non-injured controls. Recent reports have demonstrated that chronic inflammation (months to years) persists in TBI patients long after the injury. Our inflammatory panel has identified multiple inflammatory signatures in the form of upregulated adhesion molecules, interleukins, chemokines, cytokines, and other genes overexpressed in the NAc of moderately injured mice at 2 weeks post-TBI (immediately preceding the beginning of cocaine CPP). Taken together, these results suggest that the heightened response to cocaine observed in CPP testing is potentially mediated by persistent neuroinflammation post-TBI. As such, pre-clinical models of TBI may be excellent models for investigating the development of drug dependence, given that TBI may intensify the reward gained from taking drugs of abuse by a precipitating neuroinflammatory dysfunction in the corticostriatal circuit.

Acknowledgments

This work was supported by National Institutes of Health/National Institute on Drug Abuse (NIH/NIDA) T32 DA007237 (to S.F.M.) and P30 DA013429-16 (to S.M.R. and S.H.R.), NIH/National Institute of Neurological Disorders and Stroke (NINDS) R01 NS086570-01 (to S.H.R.), The Shriners Hospitals for Children 85110-PHI-14 (to S.H.R.), and NIH/National Institute of Mental Health (NIMH) R01 MH65151 (to Y.P.).

Author Disclosure Statement

No competing financial interests exist.

References

1. Faul, M., Xu, L., Wald, M.M., and Coronado, V.G. (2010). Traumatic brain injury in the United States: emergency department visits, hospitalizations and deaths 2002–2006. National Center for Injury Prevention and Control, Centers Disease Control: Atlanta, GA.
2. Eierud, C., Craddock, R.C., Fletcher, S., Aulakh, M., King-Casas, B., Kuehl, D., and LaConte, S.M. (2014). Neuroimaging after mild traumatic brain injury: review and meta-analysis. *Neuroimage Clin.* 4, 283–294.
3. Whelan-Goodinson, R., Ponsford, J., Johnston, L., and Grant, F. (2009). Psychiatric disorders following traumatic brain injury: their nature and frequency. *J. Head Trauma Rehabil.* 24, 324–332.
4. Olson-Madden, J.H., Brenner, L., Harwood, J.E., Emrick, C.D., Corrigan, J.D., and Thompson, C. (2010). Traumatic brain injury and psychiatric diagnoses in veterans seeking outpatient substance abuse treatment. *J. Head Trauma Rehabil.* 25, 470–479.
5. Corrigan, J.D., Bogner, J., Mellick, D., Bushnik, T., Dams-O'Connor, K., Hammond, F.M., Hart, T., and Kolakowsky-Hayner, S. (2013). Prior history of traumatic brain injury among persons in the Traumatic Brain Injury Model Systems National Database. *Arch. Phys. Med. Rehabil.* 94, 1940–1950.
6. Ramesh, D., Keyser-Marcus, L.A., Ma, L., Schmitz, J.M., Lane, S.D., Marwitz, J.H., Kreutzer, J.S., and Moeller, F.G. (2015). Prevalence of traumatic brain injury in cocaine-dependent research volunteers. *Am. J. Addict.* 24, 341–347.
7. Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. *Brain* 136, 28–42.
8. Glushakova, O.Y., Johnson, D., and Hayes, R.L. (2014). Delayed increases in microvascular pathology after experimental traumatic brain injury are associated with prolonged inflammation, blood–brain barrier disruption, and progressive white matter damage. *J. Neurotrauma* 31, 1180–1193.
9. Mayeux, J.P., Teng, S.X., Katz, P.S., Gilpin, N.W., and Molina, P.E. (2015). Traumatic brain injury induces neuroinflammation and neuronal degeneration that is associated with escalated alcohol self-administration in rats. *Behav. Brain Res.* 279, 22–30.

10. Lowing, J.L., Susick, L.L., Caruso, J.P., Provenzano, A.M., Raghupathi, R., and Conti, A.C. (2014). Experimental traumatic brain injury alters ethanol consumption and sensitivity. *J. Neurotrauma* 31, 1700–1710.
11. Sajja, V.S., Galloway, M., Ghodoussi, F., Kepsel, A., and VandeVord, P. (2013). Effects of blast-induced neurotrauma on the nucleus accumbens. *J. Neurosci. Res.* 91, 593–601.
12. Shin, S.S., Bales, J.W., Yan, H.Q., Kline, A.E., Wagner, A.K., Lyons-Weiler, J., and Dixon, C.E. (2013). The effect of environmental enrichment on substantia nigra gene expression after traumatic brain injury in rats. *J. Neurotrauma* 30, 259–270.
13. Lynch, W.J., Nicholson, K.L., Dance, M.E., Morgan, R.W., and Foley, P.L. (2010). Animal models of substance abuse and addiction: implications for science, animal welfare, and society. *Comp. Med.* 60, 177–188.
14. Tallarida, C.S., Tallarida, R.J., and Rawls, S.M. (2015). Levamisole enhances the rewarding and locomotor-activating effects of cocaine in rats. *Drug Alcohol Depend.* 149, 145–150.
15. Abdul-Muneer, P.M., Chandra, N., and Haorah, J. (2015). Interactions of oxidative stress and neurovascular inflammation in the pathogenesis of traumatic brain injury. *Mol. Neurobiol.* 51, 966–979.
16. Ahmad, A., Crupi, R., Campolo, M., Genovese, T., Esposito, E., and Cuzzocrea, S. (2013). Absence of TLR4 reduces neurovascular unit and secondary inflammatory process after traumatic brain injury in mice. *PLoS One* 8, e57208.
17. Khan, M., Im, Y.B., Shunmugavel, A., Gilg, A.G., Dhindsa, R.K., Singh, A.K., and Singh, I. (2009). Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *J. Neuroinflammation* 6, 32.
18. Cunningham, C.L., Dickinson, S.D., Grahame, N.J., Okorn, D.M., and McMullin, C.S. (1999). Genetic differences in cocaine-induced conditioned place preference in mice depend on conditioning trial duration. *Psychopharmacology* 146, 73–80.
19. Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U. S. A.* 85, 5274–5278.
20. Hyman, S.E., Malenka, R.C., and Nestler, E.J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu. Rev. Neurosci.* 29, 565–598.
21. Nestler, E.J. (2005). Is there a common molecular pathway for addiction? *Nat. Neurosci.* 8, 1445–1449.
22. Levin, H.S., Williams, D.H., Eisenberg, H.M., High, W.M., Jr., and Guinto, F.C., Jr. (1992). Serial MRI and neurobehavioral findings after mild to moderate closed head injury. *J. Neurol. Neurosurg. Psychiatry* 55, 255–262.
23. Kalivas, P.W. (2009). The glutamate homeostasis hypothesis of addiction. *Nat. Rev. Neurosci.* 10, 561–572.
24. Crews, F.T., Zou, J., and Qin, L. (2011). Induction of innate immune genes in brain create the neurobiology of addiction. *Brain Behav. Immun.* 25, Suppl. 1, S4–S12.
25. Torres, L.H., Garcia, R.C., Blois, A.M., Dati, L.M., Durao, A.C., Alves, A.S., Pacheco-Neto, M., Mauad, T., Britto, L.R., Xavier, G.F., Camarini, R., and Marcourakis, T. (2015). Exposure of neonatal mice to tobacco smoke disturbs synaptic proteins and spatial learning and memory from late infancy to early adulthood. *PLoS One* 10, e0136399.
26. Lim, Y.W., Meyer, N.P., Shah, A.S., Budde, M.D., Stemper, B.D., and Olsen, C.M. (2015). Voluntary Alcohol Intake following Blast Exposure in a Rat Model of Mild Traumatic Brain Inj. *PLoS One* 10, e0125130.
27. Weil, Z., Karelina, K., Gaier, K.R., Corrigan, T.E., and Corrigan, J.D. (2015). Juvenile traumatic brain injury increases alcohol consumption and reward in female mice. *J. Neurotrauma* Oct 8. doi: 10.1089/neu.2015.3953. [Epub ahead of print]
28. Chen, S., and Charness, M.E. (2008). Ethanol inhibits neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling. *Proc. Natl. Acad. Sci. U. S. A.* 105, 19962–19967.
29. Martin, B.J., Naughton, B.J., Thirtamara-Rajamani, K., Yoon, D.J., Han, D.D., Devries, A.C., and Gu, H.H. (2011). Dopamine transporter inhibition is necessary for cocaine-induced increases in dendritic spine density in the nucleus accumbens. *Synapse* 65, 490–496.
30. Motzkin, J.C., Baskin-Sommers, A., Newman, J.P., Kiehl, K.A., and Koenigs, M. (2014). Neural correlates of substance abuse: reduced functional connectivity between areas underlying reward and cognitive control. *Hum. Brain Mapp.* 35, 4282–4292.
31. Wee, S., and Koob, G.F. (2010). The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology* 210, 121–135.
32. Kerner, B. (2009). Glutamate neurotransmission in psychotic disorders and substance abuse. *Open Psychiatr. J.* 3, 1–8.
33. Shah, S., Yallampalli, R., Merkley, T.L., McCauley, S.R., Bigler, E.D., Macleod, M., Chu, Z., Li, X., Troyanskaya, M., Hunter, J.V., Levin, H.S., and Wilde, E.A. (2012). Diffusion tensor imaging and volumetric analysis of the ventral striatum in adults with traumatic brain injury. *Brain Inj.* 26, 201–210.
34. Alhilali, L.M., Delic, J.A., Gumus, S., and Fakhra, S. (2015). Evaluation of white matter injury patterns underlying neuropsychiatric symptoms after mild traumatic brain injury. *Radiology* 277, 793–800.
35. Koob, G.F., and Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. *Science* 278, 52–58.
36. Goodrich, G.S., Kabakov, A.Y., Hameed, M.Q., Dhamne, S.C., Rosenberg, P.A., and Rotenberg, A. (2013). Ceftriaxone treatment after traumatic brain injury restores expression of the glutamate transporter, GLT-1, reduces regional gliosis, and reduces post-traumatic seizures in the rat. *J. Neurotrauma* 30, 1434–1441.
37. Wagner, A.K., Sokoloski, J.E., Ren, D., Chen, X., Khan, A.S., Zafonte, R.D., Michael, A.C., and Dixon, C.E. (2005). Controlled cortical impact injury affects dopaminergic transmission in the rat striatum. *J. Neurochem.* 95, 457–465.
38. Hussain, Z.M., Fitting, S., Watanabe, H., Usynin, I., Yakovleva, T., Knapp, P.E., Scheff, S.W., Hauser, K.F., and Bakalkin, G. (2012). Lateralized response of dynorphin A peptide levels after traumatic brain injury. *J. Neurotrauma* 29, 1785–1793.
39. Hinzman, J.M., Thomas, T.C., Quintero, J.E., Gerhardt, G.A., and Lifshitz, J. (2012). Disruptions in the regulation of extracellular glutamate by neurons and glia in the rat striatum two days after diffuse brain injury. *J. Neurotrauma* 29, 1197–1208.
40. Donnemiller, E., Brenneis, C., Wissel, J., Scherfler, C., Poewe, W., Riccabona, G., and Wenning, G.K. (2000). Impaired dopaminergic neurotransmission in patients with traumatic brain injury: a SPECT study using 123I-beta-CIT and 123I-IBZM. *Eur. J. Nucl. Med.* 27, 1410–1414.
41. Shimada, R., Abe, K., Furutani, R., and Kibayashi, K. (2014). Changes in dopamine transporter expression in the midbrain following traumatic brain injury: an immunohistochemical and in situ hybridization study in a mouse model. *Neurol. Res.* 36, 239–246.
42. Yan, H.Q., Kline, A.E., Ma, X., Hooghe-Peters, E.L., Marion, D.W., and Dixon, C.E. (2001). Tyrosine hydroxylase, but not dopamine beta-hydroxylase, is increased in rat frontal cortex after traumatic brain injury. *Neuroreport* 12, 2323–2327.
43. Yan, H.Q., Ma, X., Chen, X., Li, Y., Shao, L., and Dixon, C.E. (2007). Delayed increase of tyrosine hydroxylase expression in rat nigrostriatal system after traumatic brain injury. *Brain Res.* 1134, 171–179.
44. Redell, J.B., Moore, A.N., and Dash, P.K. (2003). Expression of the prodynorphin gene after experimental brain injury and its role in behavioral dysfunction. *Exp. Biol. Med.* (Maywood) 228, 261–269.
45. Wang, X., Loram, L.C., Ramos, K., de Jesus, A.J., Thomas, J., Cheng, K., Reddy, A., Somogyi, A.A., Hutchinson, M.R., Watkins, L.R., and Yin, H. (2012). Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc. Natl. Acad. Sci. U. S. A.* 109, 6325–6330.
46. Hutchinson, M.R., Northcutt, A.L., Hiranita, T., Wang, X., Lewis, S.S., Thomas, J., van Steeg, K., Kopajtic, T.A., Loram, L.C., Sfrigola, C., Galer, E., Miles, N.E., Bland, S.T., Amat, J., Rozeske, R.R., Maslanik, T., Chapman, T.R., Strand, K.A., Fleschner, M., Bachtell, R.K., Somogyi, A.A., Yin, H., Katz, J.L., Rice, K.C., Maier, S.F., and Watkins, L.R. (2012). Opioid activation of toll-like receptor 4 contributes to drug reinforcement. *J. Neurosci.* 32, 11187–11200.
47. Blednov, Y.A., Benavidez, J.M., Geil, C., Perra, S., Morikawa, H., and Harris, R.A. (2011). Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behav. Immun.* 25, Suppl. 1, S92–S105.
48. Adler, M.W., and Rogers, T.J. (2005). Are chemokines the third major system in the brain? *J. Leukoc. Biol.* 78, 1204–1209.
49. Ardel, A.A., Bhattacharyya, B.J., Belmadani, A., Ren, D., and Miller, R.J. (2013). Stromal derived growth factor-1 (CXCL12) modulates synaptic transmission to immature neurons during post-ischemic cerebral repair. *Exp. Neurol.* 248, 246–253.

50. Mithal, D.S., Ren, D., and Miller, R.J. (2013). CXCR4 signaling regulates radial glial morphology and cell fate during embryonic spinal cord development. *Glia* 61, 1288–1305.
51. Rau, T.F., Kothiwal, A.S., Rova, A.R., Brooks, D.M., and Poulsen, D.J. (2012). Treatment with low-dose methamphetamine improves behavioral and cognitive function after severe traumatic brain injury. *J. Trauma Acute Care Surg.* 73, 2 Suppl. 1, S165–S172.
52. Edut, S., Rubovitch, V., Rehavi, M., Schreiber, S., and Pick, C.G. (2014). A study on the mechanism by which MDMA protects against dopaminergic dysfunction after minimal traumatic brain injury (mTBI) in mice. *J. Mol. Neurosci.* 54, 684–697.
53. Edut, S., Rubovitch, V., Schreiber, S., and Pick, C.G. (2011). The intriguing effects of ecstasy (MDMA) on cognitive function in mice subjected to a minimal traumatic brain injury (mTBI). *Psychopharmacology* 214, 877–889.

Address correspondence to:

Servio H. Ramirez, PhD

Department of Pathology and Laboratory Medicine

Temple University School of Medicine

3500 North Broad Street

Philadelphia, PA 19140

E-mail: servio@temple.edu