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Endogenous Neuronal Replacement in the Juvenile Brain Following Cerebral Ischemia

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Abstract—Replacement of dead neurons following ischemia, either via enhanced endogenous neurogenesis or stem cell therapy, has long been sought. Unfortunately, while various therapies that enhance neurogenesis or stem cell therapies have proven beneficial in animal models, they have all uniformly failed to truly replace dead neurons in the ischemic core to facilitate long-term recovery. Remarkably, we observe robust repopulation of medium-spiny neurons within the ischemic core of juvenile mice following experimental stroke. Despite extensive neuronal cell death in the injured striatum of both juveniles and adults at acute time points after ischemia (24 h and 7 d), mature newborn neurons replaced lost striatal neurons at 30 d post-ischemia. This neuronal repopulation was found only in juveniles, not adults, and importantly, was accompanied by enhanced post-ischemic behavioral recovery at 30 d. Ablation of neurogenesis using irradiation prevented neuronal replacement and functional recovery in MCAo-injured juvenile mice. In contrast, findings in adults were consistent with previous reports, that newborn neurons failed to mature and died, offering little therapeutic potential. These data provide support for neuronal replacement and consequent functional recovery following ischemic stroke and new targets in the development of novel therapies to treat stroke. **Published by Elsevier Ltd on behalf of IBRO.**

Key words: cerebral ischemia, endogenous recovery, neural stem cells, neurogenesis, neuron replacement.

INTRODUCTION

Approximately 800,000 Americans experience a new or recurrent stroke every year, and many survivors continue to live with permanent stroke-related disabilities, often leading to poor quality of life (Korda and Douglas, 1997; Mercier et al., 2001; Sun et al., 2014). Although stroke is one of the leading causes of death and disability worldwide, no successful long-term neuroprotective therapies have been found in clinical trials to date (Kidwell et al., 2001; Ginsberg, 2008; Minnerup et al., 2012), highlighting the need for novel therapeutic approaches. Neuronal replacement could result in direct recovery of function, since many post-ischemic impairments are due to neuronal damage or death. Neurogenesis (the birth of new neurons) is one emerging approach involving the generation of functionally integrated neurons from progenitor cells, and occurs throughout life in the brain of mammals, making it an appealing target for potential interventions to enhance post-stroke recovery.

Long-standing evidence indicates that cerebral ischemia initiates adult neurogenesis (Liu et al., 1998; Jin et al., 2001; Arvidsson et al., 2002; Nakatomi et al., 2002). Stroke-induced neurogenesis in adult mice involves vigorous proliferation and migration of neural progenitor cells, but most cells die within 4 weeks (Lichtenwalner and Parent, 2006; Liu et al., 2013; Tobin et al., 2014), unable to repair tissue and repopulate damaged areas (Zhao et al., 2008). The timeline for rapid proliferation is short-lived, peaking at 7 days post-injury (Lichtenwalner and Parent, 2006; Liu et al., 2013). Much research has focused on neonatal, perinatal, and adult rodents, yet few studies have assessed post-stroke neurogenesis in juveniles. Research centered on adult neurogenesis in rodent models of cerebral ischemia demonstrates little replacement of neurons lost following stroke-induced damage. Adult neurogenesis differs from developmental neurogenesis, where the brain is undergoing processes

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such as axon pathfinding, programmed cell death, and dendritic extension, which are limited in the mature neurons of the adult brain (Danzer, 2008). The juvenile brain is ideal for studying neurogenesis because 3–4-week-old mice have a fully developed brain that has reached neuronal maturity like adults, yet isn't vulnerable to the developmental processes found in the neonatal and perinatal brain. Differences between the juvenile and adult brain may shed light on potential interventions that could be used to enhance neurogenesis in adults, or help identify why newborn neurons do not survive in adults.

To test the hypothesis that neurogenesis and functional recovery may be enhanced in the juvenile brain, we compared an experimental ischemic stroke (Herson et al., 2013) model in juvenile and adult mice. We examined neurogenesis and neuronal replacement in the striatum with neurobehavioral assays of functional recovery and immunohistochemistry, including bromodeoxyuridine (BrdU) labeling and cell-type specific markers at 24 h, 7 d, and 30 d following 45-min transient middle cerebral artery occlusion (MCAo). The vast majority of neurons lost in the striatum following stroke are GABAergic medium-sized spiny neurons (MSN), which are the primary neuronal type (90–95%) in the region and are essential for motor function (Arlotta et al., 2008). To determine if striatal neurogenesis plays a role in post-stroke recovery of function, we tested an array of motor and locomotive functions at baseline, 7 d, and 30 d after cerebral ischemia.

Despite equivalent injury between age groups at acute time points (24 h and 7 d), we discovered a robust regenerative response in the juvenile brain at 30 d post-injury not found in adults. We found substantial neuronal replacement in areas of ischemic damage unique to the juvenile brain, along with improved functional outcomes on behavioral tests, revealing improved limb use and motor responses in MCAo-injured juvenile mice, but not adults.

EXPERIMENTAL PROCEDURES

Seventy-two male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were randomly assigned to one of two groups for molecular experiments (MCAo Adult or MCAo Juvenile), one of four groups for behavior tests (MCAo Adult, Sham-operated Adult, MCAo Juvenile, or Sham-operated Juvenile), and the irradiation experiment included MCAo Juvenile + irradiation mice. Mice were single housed, in temperature- (23 ± 3 °C) and light (12:12 h, light:dark)-controlled rooms with ad libitum access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory animals in biological research.

Middle cerebral artery occlusion (MCAo)

MCAo methods are as previously reported (Jia et al., 2011; Herson et al., 2013). Briefly, cerebral ischemia was induced under isoflurane anesthesia in juvenile (postnatal day 20–25, 10–15 g) and adult (8 weeks, 25–30 g) mice for 45 min with reversible MCAo via the intraluminal suture method. Minor variations were incorporated to accommodate the small size of P20-25 mice (a 6–0 nylon suture was heat-blunted and coated with silicone gel to obtain a smaller filament diameter of ~0.18 mm). The adequacy of MCAo was confirmed by laser Doppler flowmetry (>70% drop required for inclusion) measured over the ipsilateral parietal cortex in all mice.

Bromodeoxyuridine (BrdU) administration

Two injections of BrdU (50 mg/kg in 0.9% saline, i.p.; Sigma, St. Louis, MO, USA) were given at 24 h and 48 h after stroke, at peak expression times reported in the literature following stroke. A synthetic analog of thymidine, BrdU is commonly used in the detection of proliferating cells in living tissues.

Immunohistochemistry

Tissue collection, staining, and analyses were performed by a blinded investigator. Cellular proliferation and neurogenesis was assessed by BrdU co-localization with cell type-specific markers, since developing neurons express distinct markers during the maturation process. Immunofluorescence assays also included markers for GABAergic MSNs, the primary neuronal type in the striatum (90–95%), and for the remaining neuronal types (5–10%), cholinergic interneurons and GABAergic parvalbumin-immunoreactive interneurons (Chang and Kita, 1992; Arlotta et al., 2008). Staining of 50-μm sections consisted of phosphate-buffered saline washes (1× PBS, 3 × 5 min), 1-h incubation in blocking serum (5% normal donkey serum with 0.3% Triton X-100), overnight incubation at 4 °C in primary antibody, PBS washes (3 × 5 min), 1-h incubation in secondary antibody, PBS washes (3 × 5 min), Hoechst counterstain 5 min (1:10,000 in PBS), PBS washes (3 × 5 min), mount and coverslip with anti-fade mounting medium (Vectashield). For BrdU staining, sections were washed with 1× PBS (3 × 5 min), denatured (2 N HCl) for 20 min at 37 °C, neutralized with 0.1 M borate buffer (pH 8.5, 3 × 15 min), PBS washes (3 × 5 min), and finished using the protocol listed above. The following primary antibodies for cell-specific markers were used: rat anti-BrdU (1:300, Abcam), rabbit anti-doublecortin (DCX, 1:500, Abcam), mouse anti-NeuN (1:500, Millipore), rabbit anti-COUP-TF-interacting protein 2 (Ctip2, 1:300, Abcam), rat anti-Ctip2 (1:300, Abcam), rabbit anti-choline acetyltransferase (ChAT, 1:300, Millipore), mouse anti-parvalbumin (PV, 1:300, Sigma), goat anti-glial fibrillary acidic protein (GFAP, 1:500, Santa Cruz Biotech), and rabbit anti-oligodendrocyte transcription factor 2 (Olig2, 1:300, Millipore). The following secondary antibodies were used: Alexa Fluor 488, 594, or 647-conjugated IgG (1:500 or 1:600; Jackson Immuno) and Alexa Fluor 555 (1:500, Abcam). Cell death was assessed by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, Cell Death Detection Kit, Roche) assay. Confocal microscopy was used to confirm co-localization of BrdU and cell type-specific markers using an Olympus FV1000 laser scanning confocal microscope and

Olympus Fluoview imaging software (Center Valley, PA, USA). The Cell Counter plug-in on Fiji software (Schindelin et al., 2012) was used for cell count analyses of the lateral striatum, averaged across two sections per animal with 100-μm spacing between.

Neurobehavioral testing

Stroke is commonly associated with sensory and motor deficits, including loss of coordination and partial paralysis (Mercier et al., 2001), and a leading goal of stroke treatment is the restoration of behavioral function to patients. Investigation of neurobehavioral recovery in juvenile compared to adult mice following cerebral ischemia will help to determine the functional significance of neurogenesis in enhanced post-stroke outcomes. Damage to the forelimb region of sensorimotor cortex causes deficits in limb and motor function that can be assessed with simple tasks that are not affected by repeated testing, do not require training or aversive motivation, and have been validated in unilateral models of stroke in rodents measuring post-ischemic recovery of function (Bland et al., 2000; Schallert et al., 2000; Hua et al., 2002; Zhang et al., 2002; Woodlee et al., 2005; Schaar et al., 2010). Multiple measures were chosen to evaluate different aspects of motor and sensory responses, which may have been undetected by a single test. Behavioral assessment was performed by a blinded investigator, and included a battery of tests (Table 1) measured at baseline, 7 d and 30 d following MCAo in juvenile, adult, and sham-operated controls.

Table 1. Neurobehavioral tests

Behavioral test	Scoring	References
Vibrissae-elicited forelimb placing test: detects impairments in visual placing and limb use asymmetry	The observed ability of ipsilateral (right) and contralateral (left) placing of the forepaw on top of the table was scored as: 0 = no attempt to place forepaw, 1 = weak attempt to place forepaw, or 2 = normal placing of forepaw. Scores were averaged across four consecutive trials of each limb and converted to percent placing	Markgraf et al. (1992), Schallert et al. (2000), Hua et al. (2002), Woodlee et al. (2005)
Spontaneous forelimb use: used to assess walking score, general limb use, and limb neglect	The observed use of ipsilateral and contralateral forelimbs and hindlimbs, were scored and averaged as: 0 = no movement of limb; 1 = barely perceptible movement of limb; 2 = movement, but limb does not support weight; 3 = limb supports weight and animal takes a few steps; 4 = animal walks with mild paresis; 5 = normal limb use, no detectable deficits. Scores were averaged across four consecutive trials of each limb and converted to percentage limb use	Burkey et al. (1996), Bland et al. (2000, 2001)
Toe spread: used to assess gross motor function	Mice were elevated by the tail and the amount of toe spread observed in ipsilateral and contralateral forelimbs and hindlimbs were scored as: 0 = no spreading; 1 = intermediate spreading; 2 = sustained spreading of all toes. Scores were averaged across four consecutive trials of each limb and converted to percent toe spread	Nitz et al. (1986), Brenneis et al. (2013)
Catalepsy grid test: used to assess rigid/cataleptic body postures	A wire grid 28 × 14 cm was tilted at a 45° angle inside a testing chamber and mice were placed on the grid facing down. The time before mice begin to move downward or turn and face upward (negative geotaxis) on the grid was recorded in seconds across a 2 min observation period	Fuenmayor and Vogt (1979), Saposnik et al. (1999)
Open field test: used to assess gross motor and exploratory locomotive behavior	Open field locomotor activity was assessed for 15 min in a circular open field (60 cm arena diameter, 30 cm wall height). Horizontal locomotion data was obtained with ANY-maze automated tracking software (Stoelting, Wood Dale, IL), total distance traveled and speed were recorded during spontaneous exploration	Brooks and Dunnett (2009), Seibenhener and Wooten (2015)
Corner test: used to assess sensory–motor deficits and postural asymmetry	Mice were placed in a Plexiglas chamber, with two walls (30 × 20 × 1 cm^3), attached at a 30° angle with an opening along the joint to encourage corner entry. When vibrissae are stimulated upon entry into the corner, mice would rear along the wall and exit the corner, turning either right or left. Non-ischemic mice turn either left or right, but ischemic mice preferentially turn toward the non-impaired, ipsilateral (right) side. The number of left and right turns was recorded for 10 trials and score was calculated as percentage of right turns; turns in the absence of vertical rearing were not scored	Zhang et al. (2002), Balkaya et al. (2013)

General motor and limb use asymmetry measures

Forelimb placing. The vibrissae-elicited forelimb placing test is commonly used to assess deficits in visual placing and limb use asymmetry following stroke, damage to the motor system will elicit forelimb placing impairments (Markgraf et al., 1992; Schallert et al., 2000; Hua et al., 2002; Woodlee et al., 2005).

Spontaneous forelimb use. The use of the forelimbs was assessed in freely moving mice during exploratory activity in a clear, Plexiglas cube (20 × 20 × 20 cm) for 3 min to assess walking score, general limb use, and limb neglect after stroke (Burkey et al., 1996; Bland et al., 2000, 2001).

Toe spread. This test has been used to detect impairments in gross motor function (Nitz et al., 1986; Brenneis et al., 2013).

Composite score. A general measure of overall stroke-induced motor function impairment, highly correlated tests (r = 0.812–0.964) were combined (equally weighted, scores ranged from 0% to 100%) to form a motor construct across different aspects of limb use, motor response, and visual/vestibular function.

Corner test. The corner test is commonly used to assess sensory-motor deficits and postural asymmetry in rodent models of stroke, and has sensitivity in detecting sensory-motor symmetry impairments at early and late time points post-ischemia (Zhang et al., 2002; Balkaya et al., 2013).

Open field. This task was used to assess gross motor and exploratory locomotive behavior (Brooks and Dunnett, 2009; Seibenhener and Wooten, 2015).

Catalepsy grid test. Characterized by a tendency to maintain postures and rigidity of the body, catalepsy can occur after stroke (Saposnik et al., 1999), which could confound other behavioral measures.

Irradiation. In order to investigate the possible role of juvenile neurogenesis on enhanced post-stroke behavioral outcomes, we arrested neurogenesis in MCAo-injured juveniles with ionizing irradiation and assessed neuromotor outcomes at the following time points after irradiation: post-irradiation baseline, MCAo + irradiation 7 d, and MCAo + irradiation 30 d. A γ-emitting irradiator was used to arrest neurogenesis in juvenile mice at PN15. Following a 3-d recovery, baseline behavioral testing was conducted at PN18, then MCAo was delivered at PN20-25. We performed whole body irradiation, delivered in a single dose (5 Gy), based on minimum radiation exposure dosages reported for peak reductions in proliferating SVZ cells and immature neurons in a comprehensive dose–response investigation of adult neurogenesis (Mizumatsu et al., 2003). Irradiation was performed at the University of Colorado, using a γ-emitting irradiator, which allows for deep tissue irradiations with remarkable accuracy.

Statistics: All molecular and behavioral analyses were performed by a blinded investigator. For percent expression and mean cell counts, independent samples t-tests were utilized to assess differences in the injured striatum of the juvenile versus adult brain at 24 h, 7 d, and 30 d post-ischemia. One-way ANOVAs were used to compare the ipsilateral versus contralateral striatum of the juvenile versus adult brain at 24 h, 7 d, and 30 d post-ischemia. A mixed-design ANOVA (between-subjects factor: surgical group and within-subjects factor: time) and simple main effects analysis with Bonferroni correction was used to assess differences in behavioral scores of MCAo versus sham mice at baseline, 7 d, and 30 d post-injury. A repeated-measures ANOVA with Bonferroni correction for multiple comparisons was used to compare differences in behavioral scores of juvenile MCAo + Irradiation mice across baseline, 7 d, and 30 d post-injury. Data were analyzed with IBM SPSS Statistics (Armonk, NY, USA) and differences with a p-value of <0.05 were considered significant; data represent mean ± SEM.

RESULTS

Equivalent injury at acute time points

At 24 h following stroke, equivalent injury was found in the injured striatum of MCAo-injured juvenile and adult mice. No differences (p = 0.254) were found in cell death (total $TUNEL^+$ cells, marker of apoptotic signaling cascades) between juvenile 206 ± 41 and adult 270 ± 33 (Fig. 1A–D), neuronal loss ($NeuN^+$ cells, p = 0.743) between juvenile 314 ± 35 and adult 337 ± 59 (Fig. 1A–D), or neuronal cell death (%$NeuN^+$/$TUNEL^+$, p = 0.943) between juvenile 59.6 ± 15.1% and adult 60 .9 ± 9.5% (Fig. 1C, E) mice. The contralateral hemisphere of both MCAo-injured juvenile and adult mice had fewer $TUNEL^+$ ($p < 0.001$) and $NeuN^+$/$TUNEL^+$ cells ($p < 0.001$), and more $NeuN^+$ cells ($p < 0.001$) compared to the injured, ipsilateral hemisphere.

Newborn neuron survival and replacement in juveniles at chronic time points

In contrast to equivalent neuronal loss and comparable production of newborn neurons in juvenile and adult mice at acute time points, at 30 d post-ischemia we discovered striking differences between groups (Fig. 2A–E). Incredibly, neurons lost after ischemia appear to be replaced in the juvenile brain (Fig. 2A, C), but not in adults (Fig. 2B, D). The total number of mature neurons ($NeuN^+$, Fig. 2D) was greater (p = 0.003) in the injured striatum of juvenile 239 ± 45 mice compared to adults 42 ± 9, and juveniles had more 36. 9 ± 4.8% mature newborn neurons ($NeuN^+$/$BrdU^+$, Fig. 2E) than adults 9.4 ± 5.5% (p = 0.003); revealing a marked increase at P30 in newborn neurons that migrated, matured, and replaced those lost in the injured juvenile striatum compared to adult. The ipsilateral hemisphere of both MCAo-injured juvenile and

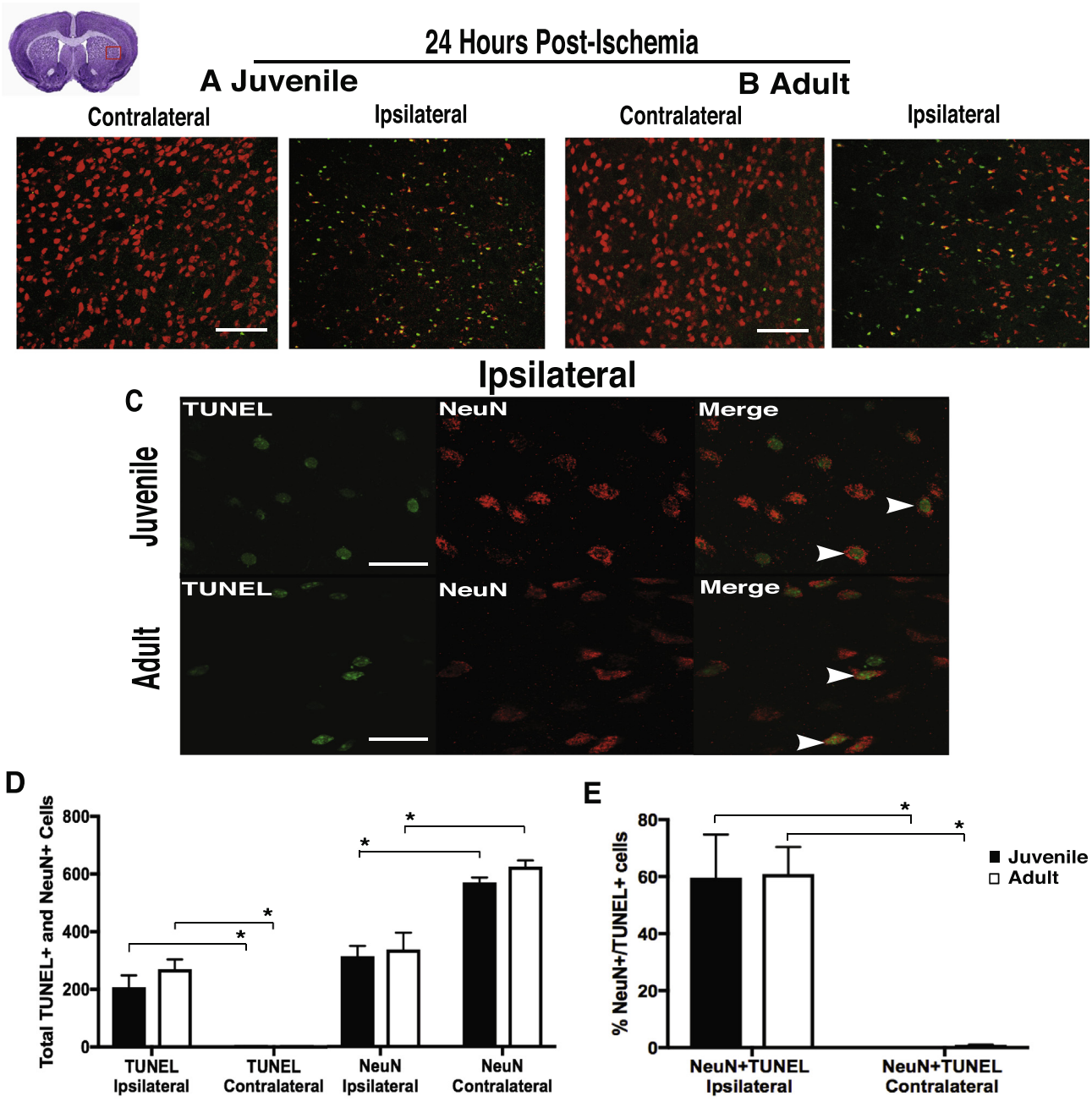

Fig. 1. Equivalent injury following ischemic stroke. (A, B) Representative images of FOV for analysis of TUNEL$^+$ (green) and NeuN$^+$ (red) cells, showing equivalent cell death and neuronal loss in the injured striatum of juvenile and adult mice at 24 h post-ischemia, compared to uninjured (contralateral) striatum. (C) Representative images at 100× magnification. (D) Comparable cell death (TUNEL$^+$), neuron loss (NeuN$^+$), and (E) neuronal cell death (NeuN$^+$/TUNEL$^+$) were found in the injured striatum of both groups. Data represent mean ± SEM, scale (A, B) = 100 μm, and scale (C) = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adult mice had more DCX$^+$ cells (<0.001) and fewer NeuN$^+$ cells ($p < 0.001$) compared to the non-injured contralateral hemisphere. There was a higher percentage of co-localized NeuN$^+$/BrdU$^+$ cells ($p < 0.001$) found in the ipsilateral hemisphere compared to contralateral, but post hoc analysis revealed that only juveniles had a higher percentage of NeuN$^+$/BrdU$^+$ cells ($p < 0.001$) in the injured hemisphere over non-injured, while co-localization in adults did not differ between hemispheres ($p = 0.343$).

MSN differentiation and replacement

To determine if newborn neurons were region-specific medium spiny neurons (MSN), co-labeling of COUP-TF-interacting protein 2 (Ctip2, marker of MSNs) and BrdU was assessed (Fig. 3A–E). At 30 d post-ischemia there were more mature neurons 219 ± 38 ($p = 0.002$) and MSNs 196 ± 54 ($p = 0.014$) in the injured hemisphere of the juvenile brain (Fig. 3D) compared to adult (24 ± 44 and 23 ± 62, respectively). There were also

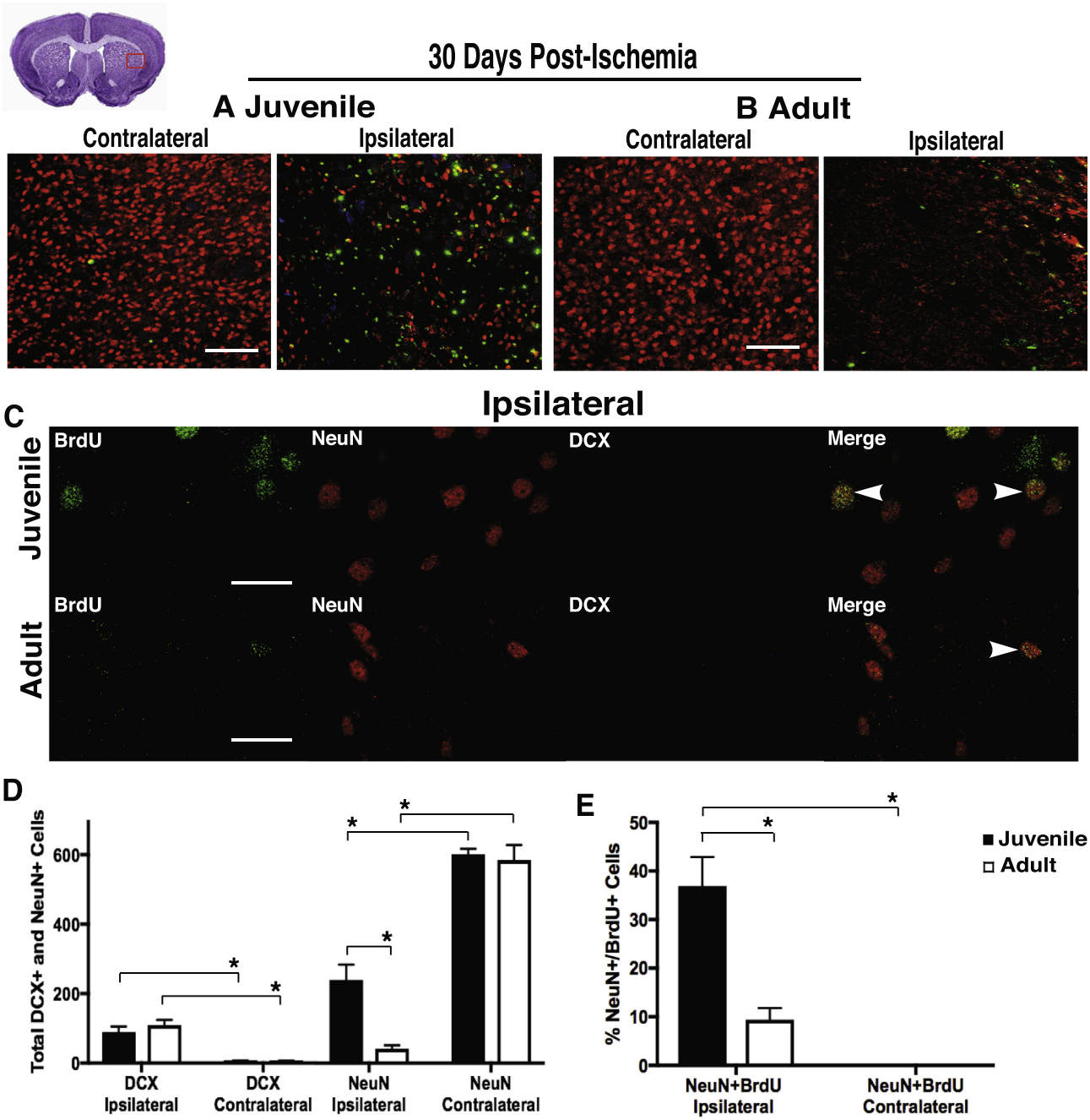

Fig. 2. Neuronal replacement after ischemic stroke. (A, B) Representative images of FOV for analysis of $BrdU^+$ (green), $NeuN^+$ (red), and DCX (blue), showing neuronal replacement in juvenile mice, but not adult at 30 d following stroke. (C) Representative images of lateral striatum at 100× magnification. (D) Juveniles had more mature neurons in the injured striatum compared to adult. (E) A marked increase in neuronal replacement in the juvenile brain, compared to adult. Data represent mean ± SEM, scale (A, B) = 100 μm, and scale (C) = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

more ($p = 0.030$) newly generated MSNs ($Ctip2^+$/$BrdU^+$, Fig. 3E) found in the injured hemisphere of juveniles 25.8 ± 6.4% compared to adult 5.1 ± 6.9% at 30 d post-stroke. Mature newborn neurons ($NeuN^+$/$BrdU^+$) were also increased ($p = 0.038$) in the ipsilateral hemisphere of juveniles 32.6 ± 7.5% (Fig. 3E) compared to adult 7.1 ± 8.0%, verifying earlier findings (Fig. 2). The contralateral hemisphere of both MCAo-injured juvenile and adult mice had more $Ctip2^+$ ($p < 0.001$) and $NeuN^+$ cells ($p < 0.001$) compared to the injured hemisphere. A higher percentage of co-localized $Ctip2^+$/$BrdU^+$ cells ($p = 0.003$) and $NeuN^+$/$BrdU^+$ cells ($p = 0.001$) was found in the ipsilateral hemisphere compared to contralateral, but post hoc analysis revealed that only juveniles had a significantly higher percentage of $Ctip2^+$/$BrdU^+$ cells ($p = 0.002$) and $NeuN^+$/$BrdU^+$ cells ($p = 0.002$) in the injured hemisphere compared to non-injured, while co-localization in adults did not differ between hemispheres ($p = 0.989$ and $p = 0.959$, respectively).

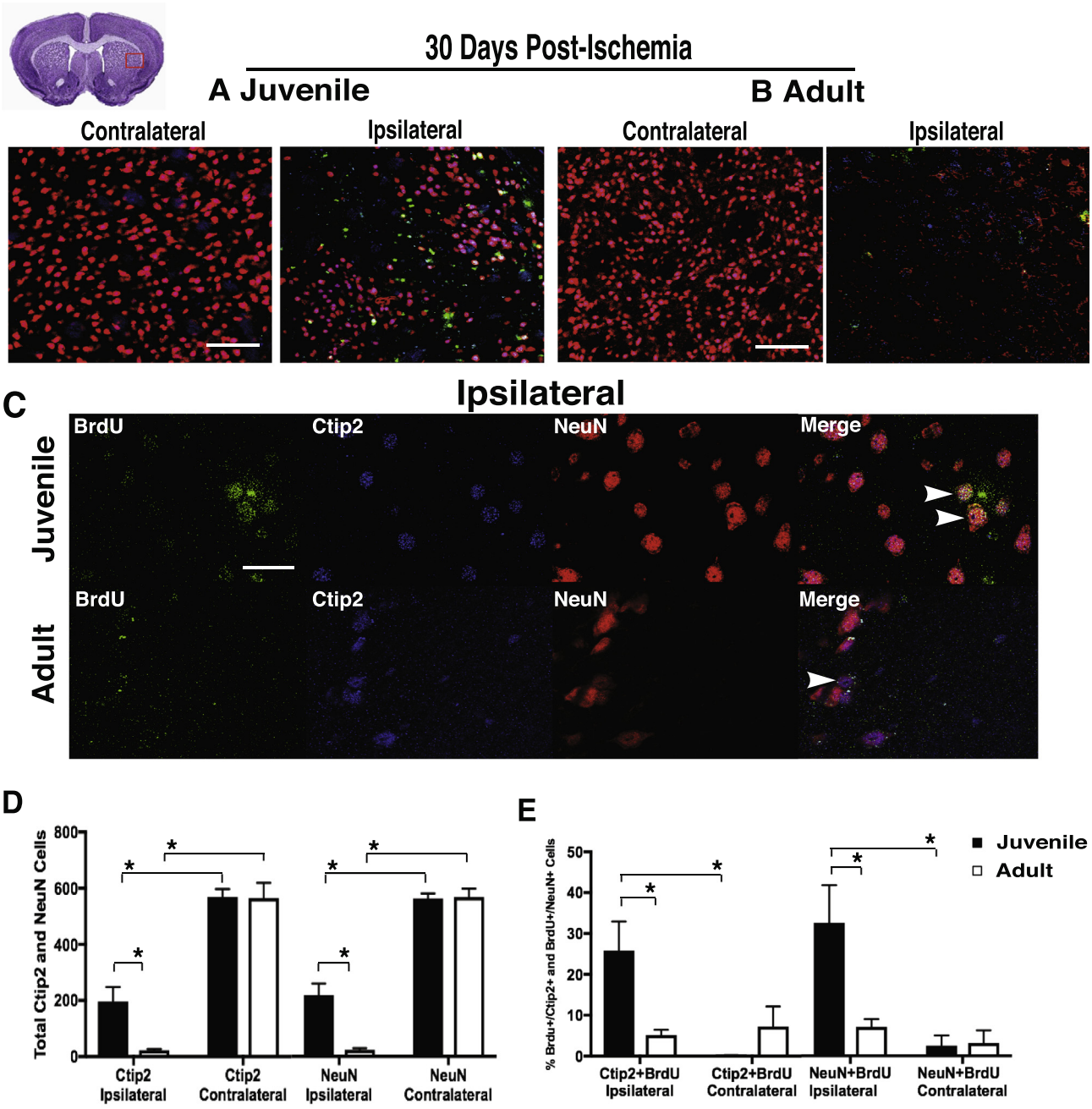

Fig. 3. MSNs after ischemic stroke. (A, B) Representative images of FOV for analysis of BrdU$^+$ (green), Ctip2$^+$ (blue), and NeuN$^+$ (red), showing that newborn neurons differentiate into region-specific MSNs in juveniles, but not adults 30 d post-ischemia. (C) Representative images of lateral striatum at 100× magnification. (D, E) Juvenile mice had more MSNs overall, and more newborn MSNs than adults. Data represent mean ± SEM, scale (A, B) = 100 μm, and scale (C) = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Distribution of striatal neuron types

Striatal cell types were examined using markers for GABAergic (Parvalbumin, PV) and Cholinergic (choline acetyltransferase, ChAT) interneurons, and the MSN marker Ctip2. At 30 d post-ischemia there were more (p = 0.014) MSNs in the injured hemisphere of the juvenile brain 184.8 ± 12.2 (Fig. 4D) compared to adult 44.7 ± 14.5. Findings showed a normal distribution of MSNs 92.2 ± 2.7% in ipsilateral striatum of juvenile mice at 30 d post-ischemia (Fig. 4A–E). This is consistent with reports of healthy striatum, which has approximately 90–95% MSNs and 5–10% GABAergic and Cholinergic interneurons (Arlotta et al., 2008). Given the extensive tissue damage sustained from ischemia, the distribution data in adult mice were not very meaningful. In spite of the distribution data and neuronal repopulation of damaged brain regions, it could be argued that BrdU is labeling DNA repair in post-mitotic neurons or

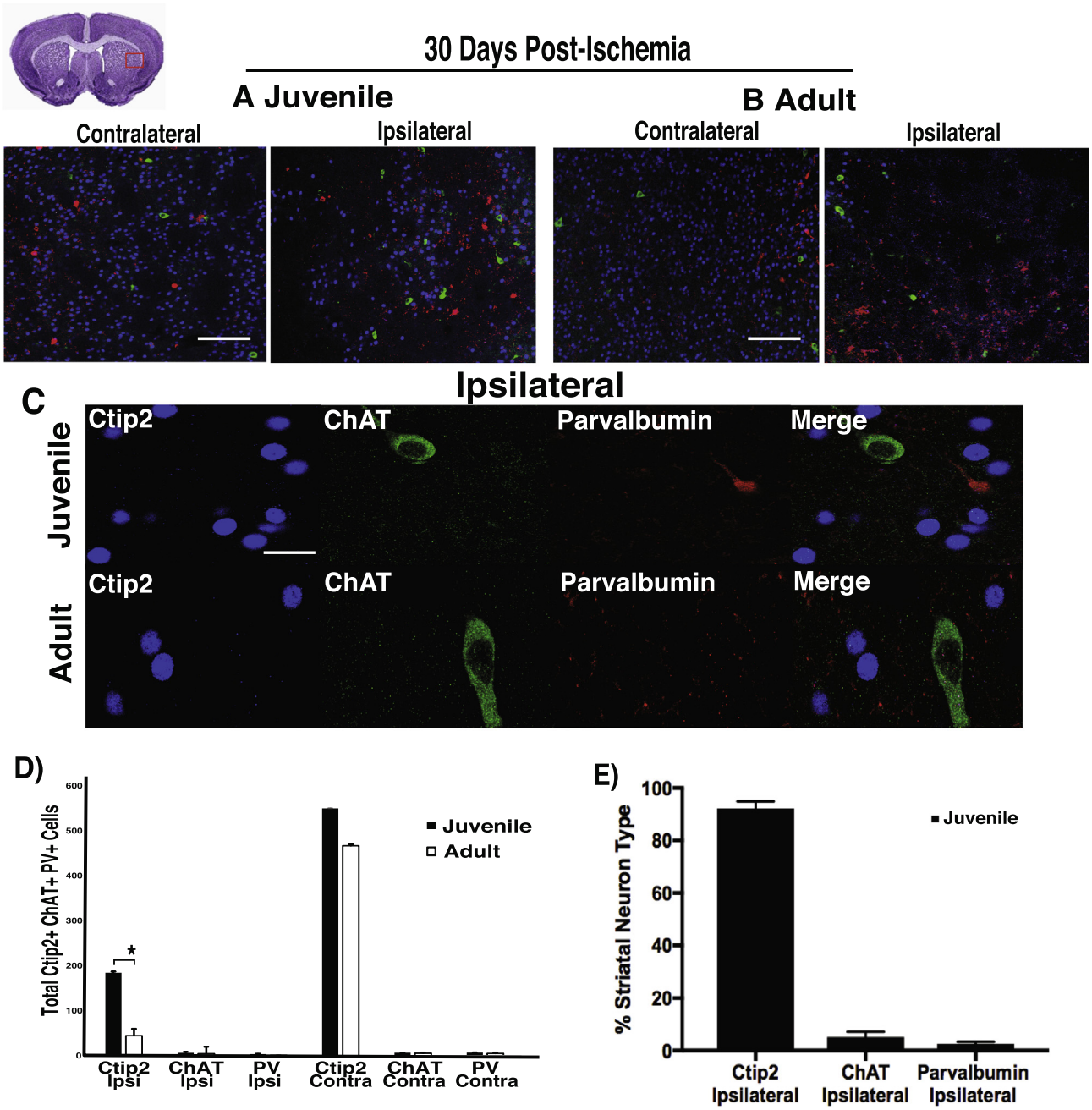

Fig. 4. MSN distribution after ischemic stroke. (A, B) Representative images of FOV for analysis of Ctip2$^+$ (blue), ChAT$^+$ (green), and Parvalbumin$^+$ (red), showing striatal cells in juveniles and adults. (C) Representative images at 100× magnification. (D, E) Juvenile mice had more MSNs in the injured striatum than adults, and the distribution of striatal neuron types in recovering juveniles was consistent with reports in healthy tissue. Data represent mean ± SEM, scale (A, B) = 100 μm, and scale (C) = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aberrant cell cycle reentry rather than neurogenesis (Cooper-Kuhn and Kuhn, 2002; Kuan et al., 2004), so we performed additional staining with BrdU and TUNEL. We did not find significant co-localization of BrdU$^+$/TUNEL$^+$ cells at 24 h and 7 d post-injury (*data not shown*), indicating that DNA repair does not account for BrdU$^+$ cells found following stroke. In order to further examine the cellular environment, an additional assay was performed including BrdU, GFAP (marker of astrocytes), and Olig2 (lineage marker of oligodendrocytes) to assess glial cell differentiation. Results did not reveal significant co-labeling of either BrdU$^+$/GFAP$^+$ cells or BrdU$^+$/Olig2$^+$ cells (*data not shown*), indicating that most newly generated cells in the injured striatum were neurons.

Neurobehavioral outcomes

The analysis revealed significant reductions in use of the affected limb, motor coordination, and general motor

functioning for MCAo-injured adults and juveniles at 7 d post-injury, with only the juveniles returning to near baseline levels on most tasks by 30 d. Sham-operated controls showed no neurological deficits or changes in behavior from baseline and are not included in the respective figures. There were no significant differences between stroke and sham-operated groups on the catalepsy grid task ($p = 0.512$), or measures of horizontal locomotion: distance traveled ($p = 0.099$) and speed ($p = 0.103$) in the open field across time points, indicating that MCAo-injured mice did not have impaired ability to initiate movement or in general locomotion, which may have confounded other behavioral measures.

On the forelimb placing task (Fig. 5A), we found reductions ($p = 0.002$) in the ability to place the affected limb for both adult 19.2 ± 5.9% ($p = 0.006$) and juvenile 25.8 ± 5.5% ($p < 0.001$) stroke-injured mice at 7 d post-injury compared to baseline. However, by the 30 d, impairment in adults 15.4 ± 3.0% ($p < 0.001$) continued, while juveniles demonstrated no deficits and had returned to baseline levels of functioning ($p = 1.000$). Comparison of 7 d and 30 d in MCAo-injured adults and juvenile mice revealed no differences in forelimb placing deficits between groups at 7 d post-injury ($p = 1.000$), but at 30 d, juveniles had greater recovery of limb placing compared to adults ($p = 0.003$). Forelimb placing in MCAo-injured adult mice did not differ between 7 d and 30 d time points post-ischemia, while juveniles had recovery of placing in the affected forelimb from 7 d to 30 d ($p = 0.000$). Further, at 30 d following stroke, only adult MCAo-injured mice significantly differed from sham-operated controls ($p = 0.008$), while juveniles had returned to similar levels of placing as sham-operated controls ($p = 1.000$). On the forelimb limb use task (Fig. 5B) we found reductions in limb use ($p = 0.006$), adult 14.6 ± 2.9% ($p < 0.001$) and juvenile 9.7 ± 2.7% ($p = 0.003$) MCAo-injured mice used the affected, contralateral forelimb less at 7 d compared to baseline. However, at 30 d post-injury only the MCAo-injured adult mice 12.3 ± 1.8% ($p = 0.000$) had continued deficits in limb use, while juveniles no longer differed from baseline limb function 2.7 ± 1.7% ($p = 0.345$). At 30 d only adult MCAo-injured mice significantly differed from sham-operated controls ($p = 0.000$), while MCAo-injured juveniles displayed recovery of forelimb function ($p = 1.000$). The composite score showed the same stroke-induced deficits at 7 days in both juvenile and adult mice that improved only in the juveniles, with no observed improvement of stroke-induced deficits in the adults (Fig. 5C). Finally, Fig. 5D shows unilateral postural bias using the corner task in both juvenile and adults that recovers specifically in the juvenile mice 30 d after recovery from MCAo.

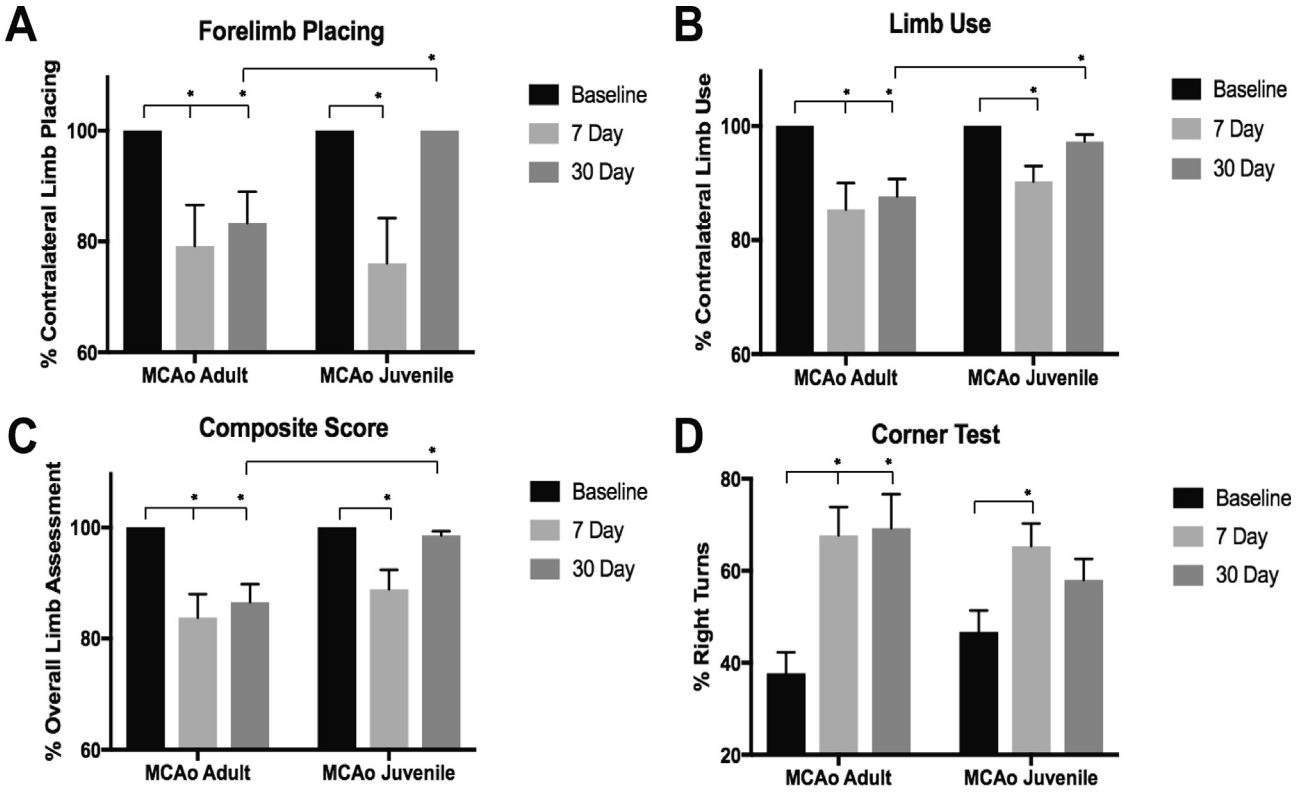

Fig. 5. Neurobehavioral outcomes after ischemic stroke. (A, B) Forelimb placing and spontaneous limb use, showing deficits in contralateral placing and limb use responses in both stroke groups at 7 d post-ischemia; however, by 30 d only the juvenile mice had returned to baseline levels, while adult mice showed continued impairment. (C) Composite score of overall impairment of the affected limb and general motor response measures, showing reduced limb function at 7 d post-injury and recovery of functioning at 30 d only in juvenile mice. (D) The corner test detected postural bias in both MCAo-injured adult and juvenile mice between baseline and 7 d, but only adult mice had extended functional impairments at 30 d.

Irradiation

On the forelimb placing task (Fig. 6A), we discovered that juvenile MCAo-injured + irradiation mice displayed reductions in the ability to place the affected forelimb at both 7 d and 30 d. We found decreased ($p = 0.001$) use of the affected forelimb in juvenile MCAo-injured + irradiation mice (Fig. 6B), at 7 d post-injury + irradiation 15.0 ± 4.2% ($p = 0.013$) and 30 d post-injury + irradiation 13.0 ± 3.8% ($p = 0.014$), demonstrating prolonged deficits in limb use compared to the recovery found at 30 d in MCAo-injured without radiation exposure, mice ($p = 1.000$). Similarly, there was no recovery of behavioral function (composite score or corner task) found between the 7-d and 30-d time points (Fig. 6D).

Irradiation and newborn neuron maturation and survival

Following irradiation newborn neurons failed to mature and survive in the injured striatum of juveniles. At 30 d post-ischemia, irradiated mice had no significant differences ($p = 0.839$) between ipsilateral and

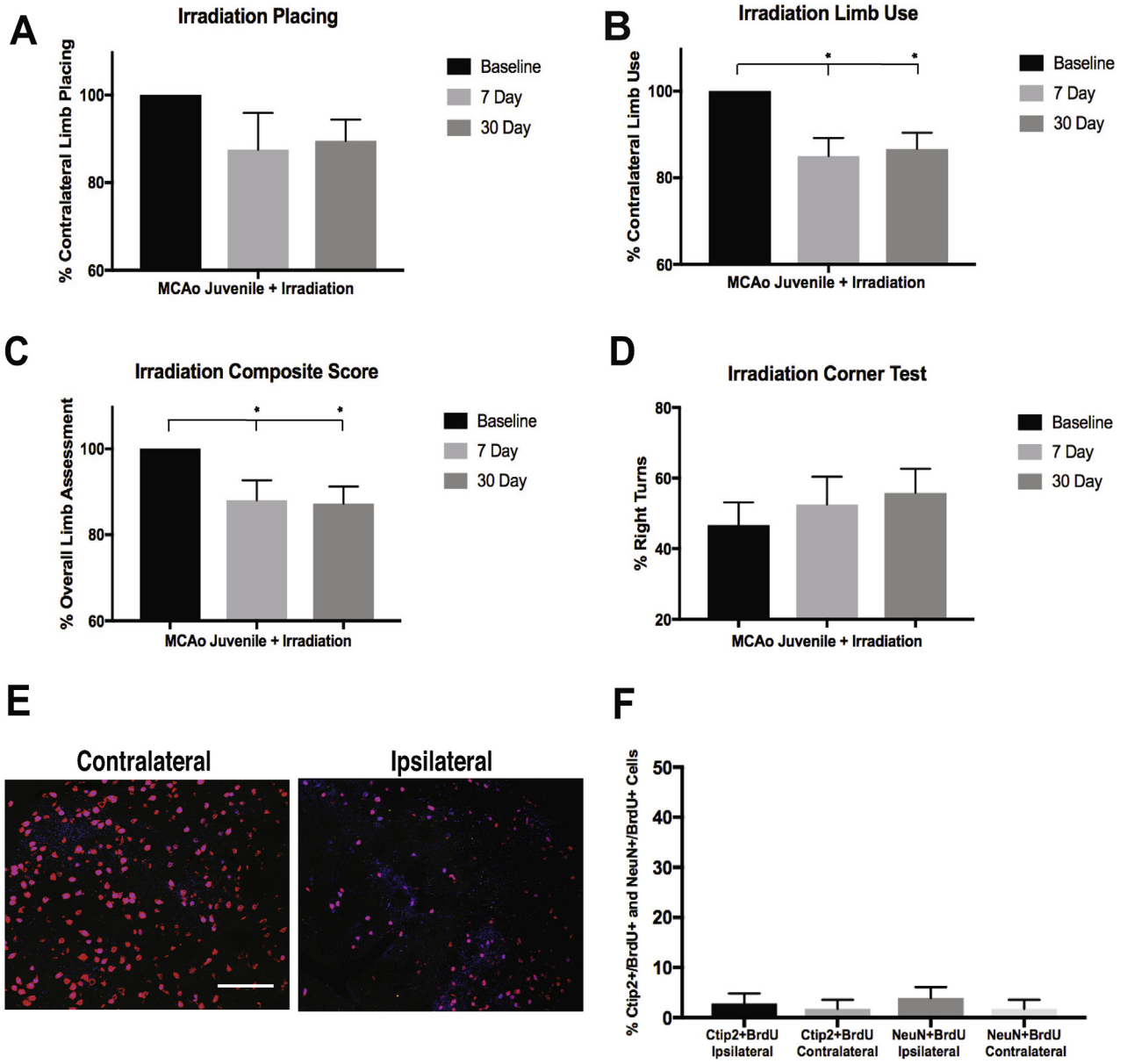

Fig. 6. Loss of functional recovery on neurobehavioral outcomes following irradiation. (A, B) Forelimb placing and spontaneous limb use, showing reduced contralateral placing responses in the MCAo + irradiation group at 7 d and 30 d, with a reversal in recovery of function at 30 d compared to non-irradiated juvenile mice. (C) On the composite measure MCAo + irradiation juvenile mice displayed decreased limb use and general motor functioning at both the 7 d and 30 d post-ischemic time points compared to baseline, with no recovery of motor function like non-irradiated mice. (D) Irradiated MCAo-injured mice had increased postural bias at 7 d and 30 d compared to baseline, showing continued deficits at chronic time points. (E-F) Representative images of FOV for analysis of $BrdU^+$ (green), $Ctip2^+$ (blue), and $NeuN^+$ (red), showing that neurogenesis (Ctip2 + /BrdU + and NeuN + /BrdU + positive cells) was abolished in the injured striatum following irradiation, and irradiation did not damage neuronal cells in the contralateral hemisphere. Data represent mean ± SEM, scale = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contralateral hemispheres (Fig. 6E, F) in the percentage newly generated MSNs ($BrdU^+/Ctip2^+$, 2.8 ± 2.0%, ipsilateral and 1.7% ± 1.8, contralateral) or mature newborn neurons ($BrdU^+/NeuN^+$, 3.9 ± 2.2% ipsilateral and 1.8% ± 1.8, contralateral). This is in sharp contrast to our earlier findings in MCAo-injured, non-irradiated mice Fig. 3E (25.8%, BrdU+/Ctip2+ and 32.6%, BrdU+/NeuN+), revealing that irradiation at 5 Gy was sufficient to arrest neurogenesis in juveniles.

DISCUSSION

Targeting neurogenesis to promote neuronal replacement and endogenous regenerative brain repair has long been intriguing, but the low survival rate of newborn neurons has left doubt about the therapeutic potential of neurogenesis. It has been widely accepted that our brains are not capable of significant self-repair and regeneration because most injury-induced newborn neurons die within 4 weeks of birth, lacking the ability to repair tissue and repopulate areas of damage (Zhao et al., 2008). Our findings show that juvenile brain appears to be capable of self-repair, as we observe a remarkable neuronal repopulation of the juvenile striatum that is coupled with improved behavioral outcomes after stroke that we do not observe in adults, providing strong evidence for enhanced neurogenesis and neuronal replacement following stroke in the injured juvenile brain.

From a clinical perspective, it is remarkable that newborn neurons migrate to the site of injury and repopulate areas where neurons have died. These observations not only increase our understanding of post-ischemic regeneration, but provide an opportunity to examine neuronal replacement following juvenile stroke as a novel therapeutic target for endogenous brain repair and recovery. These cells migrate, differentiate, and appear to replace the primary neurons lost following injury, region-specific medium spiny neurons, demonstrating the incredible potential for stroke-induced neurogenesis in the juvenile brain. This is in stark contrast with the multitude of studies in adult stroke that show a robust proliferation and migration of newborn neurons, which fail to survive within the ischemic core and die within 4 weeks (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002; Parent et al., 2002; Lichtenwalner and Parent, 2006). In addition to the cellular findings, we also discovered differences in functional outcomes, revealing several deficits on neurobehavioral tasks that recovered only in the MCAo-injured juvenile mice and not adults, highlighting the role of neurogenesis in functional recovery after stroke. Our behavioral results provide powerful evidence for improved post-ischemic outcomes, with benefits seen across a variety of measures targeting region-specific behavioral changes. Investigating neurobehavioral deficits and potential mechanisms of repair in animal models of stroke is critical for developing translational applications that could promote improved recovery of function after injury. Following stroke that affects unilateral upper extremity function, humans tend to rely on their less-affected limb, which often undermines their ability to regain function in the affected limb (Taub et al., 2006). We found impairments on several measures of limb-use function in the affected limb of both juveniles and adults at 7 d post-injury; however, we only found recovery of function in MCAo-injured juvenile mice at 30 d post-injury, while MCAo-injured adults remained consistent or exhibited increased deficits at 30 d. A sensorimotor function task (corner test) also revealed deficits in postural bias at 7 d and 30 d in MCAo-injured juvenile and adult mice, with MCAo-injured juveniles showing a reduction in right turnsacross time points compared to adults, and by 30 d post-ischemia MCAo-injured juvenile mice performing similarly to sham-operated controls.

To further investigate neurogenesis as a mechanism of neuronal replacement and recovery following stroke, we arrested neurogenesis using ionizing irradiation and assessed differences in post-ischemic behavioral recovery. Ionizing irradiation is a commonly used method of myeloablation in the mouse that causes breaks in the DNA double-strand of mitotically active cells, and this damage leads to cell death through apoptosis and necrosis (Duran-Struuck and Dysko, 2009). Neurogenesis is sensitive to irradiation in a dose-dependent fashion. Following a 5–10 Gy irradiation dose, apoptosis peaks at 12 h post-irradiation and production of new neurons is abolished at 48 h, proliferating cells are reduced by over 95% and immature migrating neuroblasts decreased by 55–70% (Mizumatsu et al., 2003; Achanta et al., 2012). Following irradiation exposure to arrest hippocampal neurogenesis, memory and cognitive impairments have been reported (Raber et al., 2004, 2011; Rola et al., 2004; Olsen et al., 2017), and following irradiation + brain injury (Rosi et al., 2012; Allen et al., 2014). While less is known about effects of irradiation on motor functioning, we found that irradiation alone was well tolerated in mice and associated with loss of recovery, similar to previous reports. When we arrested neurogenesis in MCAo-injured juveniles with a single dose (5 Gy) of ionizing irradiation, newborn neurons failed to mature and survive in the injured striatum of juveniles at 30 d post-ischemia, and the behavioral recovery previously found at 30 d was lost. In addition to diminished newborn neuron survival, irradiated juvenile mice had deficits on most tasks at 7 d after stroke. However, in stark contrast to non-irradiated mice, there was continued impairment at 30 d with no recovery observed at chronic time points. The irradiation findings implicate juvenile neurogenesis and endogenous neuronal renewal in functional recovery after stroke, supported by recovery of behavioral function only in non-irradiated MCAo-injured juvenile mice.

While the mechanisms underlying juvenile neurogenesis remain to be elucidated, it is evident that differences exist between the juvenile and adult brain, with the juvenile environment supporting neuronal survival. These findings support our previous observations of glial and white matter responses using this same model of ischemic stroke, which revealed very different glial responses and pathological sequelae after MCAo in juvenile and adult mice (Ahrendsen et al., 2016). In this study, equivalent cell death, neuronal loss, and diffuse astrogliosis were seen in the striatum of both

groups at early time points following stroke (24 h, 3 d, and 7 d); however, oligodendrocyte progenitor cells, mature oligodendrocytes, and myelinated axons were spared in juveniles while significant decreases were found in adults. At 30 d post-ischemia, dramatic differences in tissue damage were observed between juveniles and adults, with long-term preservation of brain parenchyma only observed in juvenile mice. Adults had severe axon pathology, ultrastructural damage, demyelination, glial scar formation, and major loss of striatal and cortical tissue compared to the juvenile brain, which had remarkable resistance to ischemic injury with no glial scarring and preservation of all major brain regions. These findings further define the unique environment that results in enhanced repair and recovery in the juvenile brain compared to adult.

That newborn neurons survive and repopulate stroke-damaged brain regions, and contribute to improved behavioral outcomes, is an innovative finding that supports the role of juvenile neurogenesis in neuronal replacement and enhanced functional recovery following ischemic stroke. Such studies are essential for understanding the role of neurogenesis as an intrinsic mechanism of brain repair and recovery. Newborn neuron production, maturation, survival, and replacement in the developing brain has exciting implications for promoting post-stroke neurogenesis and functional recovery in adults and children alike.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Reversal of Established Traumatic Brain Injury-Induced, Anxiety-Like Behavior in Rats after Delayed, Post-Injury Neuroimmune Suppression

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Abstract

Traumatic brain injury (TBI) increases the risk of neuropsychiatric disorders, particularly anxiety disorders. Yet, there are presently no therapeutic interventions to prevent the development of post-traumatic anxiety or effective treatments once it has developed. This is because, in large part, of a lack of understanding of the underlying pathophysiology. Recent research suggests that chronic neuroinflammatory responses to injury may play a role in the development of post-traumatic anxiety in rodent models. Acute peri-injury administration of immunosuppressive compounds, such as Ibudilast (MN166), have been shown to prevent reactive gliosis associated with immune responses to injury and also prevent lateral fluid percussion injury (LFPI)-induced anxiety-like behavior in rats. There is evidence in both human and rodent studies that post-traumatic anxiety, once developed, is a chronic, persistent, and drug-refractory condition. In the present study, we sought to determine whether neuroinflammation is associated with the long-term maintenance of post-traumatic anxiety. We examined the efficacy of an anti-inflammatory treatment in decreasing anxiety-like behavior and reactive gliosis when introduced at 1 month after injury. Delayed treatment substantially reduced established LFPI-induced freezing behavior and reactive gliosis in brain regions associated with anxiety and continued neuroprotective effects were evidenced 6 months post-treatment. These results support the conclusion that neuroinflammation may be involved in the development and maintenance of anxiety-like behaviors after TBI.

Key words: glial cell response to injury; inflammation; traumatic brain injury

Introduction

Over 5.3 million people in the United States are living with traumatic brain injury (TBI)-related disabilities,[1] including anxiety disorders, which are among the most prevalent.[2–4]

The rates of a variety of anxiety disorders reported by patients with TBI are consistently elevated relative to general population rates,[3–5] and the risk for developing post-traumatic stress disorder (PTSD) remains elevated for years postinjury.[6–8] Because the temporal pattern of onset is variable and the etiology unclear, there are currently few interventions for the treatment of PTSD.

The ongoing inflammatory response after TBI is an emerging target for the treatment of post-traumatic anxiety. Extensive research has shown that chronic neuroinflammation continues for months to years after injury,[9–12] and evidence for chronic inflammation has been observed in a number of studies examining patients with PTSD, panic disorder, obsessive-compulsive disorder (OCD), and generalized anxiety disorder.[13–18] Glial activation may be involved in the development and maintenance of PTSD,[9–12] and mounting evidence supports the role of inflammatory processes in both TBI and anxiety disorders.

After injury, immune cells rapidly produce endogenous danger signals or ''alarmins,'' which function as potent effectors of innate defense and promote immune system activation by recruiting antigen-presenting cells (APCs) that relay and amplify the inflammatory response.[19] The resident APCs of the cenral nervous system (CNS) are microglia, which undergo marked recruitment and activation in response to danger signals,[20,21] triggering the onset of prolonged astrocytic activation through the production of proinflammatory cytokines, chemokines, and other proinflammatory mediators.[22,23]

Activated microglia are thought to contribute to the initiation and maintenance of astrogliosis, which is involved in neural cell damage and inhibition of regenerative responses through secretion of excessive neurotoxic substances, destabilization of neurotransmitter balance, disruption of synaptic connectivity, and excitotoxic neuronal death[24–28] and therefore may contribute to functional alterations of brain areas involved in post-traumatic anxiety.

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Several studies have reported increased anxiety-like behavior in rodent TBI models,[29–32] including increased conditioned[33] and unconditioned[34] fear responses to both learned and novel stimuli. TBI in rodents also increases levels of activated glial cells and proinflammatory cytokines,[34–38] and administration of these cytokines increases anxiety-like behaviors.[29–32]

The aim of the present study was therefore to determine whether neuroinflammation is associated with the long-term maintenance of post-traumatic anxiety in an animal model. We examined the efficacy of delayed, immunosuppressive treatment (with a glial cell activation inhibitor, Ibudilast) in reducing anxiety-like behaviors and TBI-induced immunological damage.

Methods

Twenty-four adult viral-free male Sprague-Dawley rats (275–325 g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature- (23 ± 3°C) and light- controlled (12-h light/dark) rooms with *ad libitum* access to food and water. All procedures were performed in accord with University of Colorado (Boulder, CO) Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to the following groups ($n = 6$/group): sham + vehicle; sham + MN166; lateral fluid percussion injury (LFPI) + vehicle; and LFPI + MN166.

Lateral fluid percussion injury

LFPI is the most commonly used animal model of TBI and has been shown to reliably replicate many of the pathological changes observed after human TBI, validating its use as a clinically relevant model of human TBI.[39] The LFPI used in this study has been described previously.[40] Briefly, LFPI rats were anesthetized with halothane (3% induction, 2.0–2.5% maintenance) and mounted in a stereotaxic frame. A 3.0-mm-diameter craniotomy was centered at 3 mm caudal to the bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. After hub implantation, rats were removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered an impact force (2.0 atmospheres; 20 ms), resulting in a moderate TBI. The injury cap was then removed, the scalp sutured, and rats were returned to their home cages for recovery. Sham-operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration

MN166 (MediciNova, San Diego, CA) is a relatively nonselective phosphodiesterase inhibitor with anti-inflammatory actions by glial cell attenuation.[41,42] Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg, subcutaneously), beginning at 30 days after LFPI. Weight was recorded before each dosing and treatment administered at the same time each day to maintain constant levels across a 24-h period. Dose selection was based on previous animal pharmacology results[43] showing MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high-dose regimens in clinical development. MN166 administered by this regimen yields plasma and CNS concentrations that are linked to molecular target actions, including, most potently, macrophage migration inhibitory factor (MIF) inhibition [44] and, secondarily, phosphodiesterases −4 and −10 inhibition.[45] The relevance of MIF inhibition in disorders of neuroimmune function, such as neuropathic pain, has recently been well demonstrated.[46]

Neuromotor tests

Baseline testing of motor, vestibular, and locomotive performance in all groups was conducted immediately before surgery and again at 1 month after injury (Fig. 1). These tests included ipsi- and contralateral assessment of fore- and hindlimb use to assess motor function, locomotion, limb use, and limb preference,[47,48] toe spread to assess gross motor response,[49] placing to assess visual and vestibular function,[50,51] catalepsy rod test to assess postural support and mobility,[52] bracing to assess postural stability and catalepsy,[53,54] and air righting to assess dynamic vestibular function.[55,56] Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). Individual test scores were summed, and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function after CNS injury in rats,[50,57] and postinjury locomotor activity was assessed through distance traveled on a running wheel; both tasks were scored for 5 min under red light (~90 lux).

Behavioral measures

The core features of anxiety in humans include feelings of apprehension or dread both with or without autonomic signs and symptoms, and in the case of post-traumatic anxiety (PTSD), also includes reexperiencing trauma, avoidant behavior, and hypervigilance.[4] The immediate shock paradigm was chosen to elicit PTSD-related traits of abnormally elevated fear responses and hypervigilance. In traditional contextual fear conditioning, rats are placed in a context and shock is delayed for a period of time, allowing for association between the contextual cues and the shock, resulting in increased freezing during later testing. However, rats that are immediately shocked upon placement in a shock chamber show no increase in freezing behavior during later testing. This phenomenon is known as the immediate shock deficit and results in failure to display contextual fear conditioning because the rats do not have time to construct a representation of the context,[58–60] indicating a lack of association between the context and shock. However, we previously found that LFPI rats show increases in freezing responses even in the absence of fear conditioning.[34] These unconditioned freezing responses may reflect pathological anxiety, which involves exaggerated fear, hypervigilance, and readiness to respond to danger or negative events,[61] because freezing behavior is part of an anticipatory response to stress or danger. Shock was chosen as the stressor because it resulted in anxiety-like freezing behavior, which is a simple reproducible response elicited as a defense reaction in both conditioned and unconditioned fearful situations.[62]

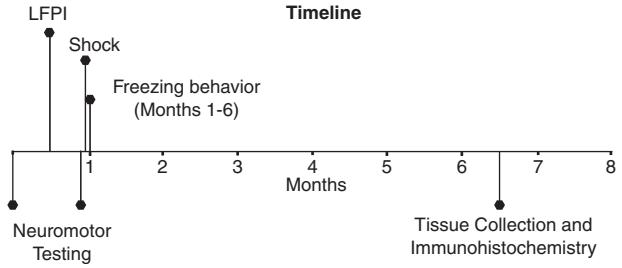

FIG. 1. Experimental timeline. Neuromotor testing included measures of motor, vestibular, and locomotive performance and was conducted immediately before LFPI and again at 1 month postinjury. A single shock was delivered after neuromotor testing was completed at the 1-month time point, and freezing behavior was assessed at 1 through 6 months postinjury. Tissue was then collected for immunohistochemistry. LFPI, lateral fluid percussion injury.

A novel environment was used for testing to ensure that there was no association between the context and shock, because rats were never tested in the context where the shock occurred. The novel context was a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. Rats were tested (5 min) and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement, except for heart beat/respiration, and was recorded in 10-sec intervals. Freezing behavior in the novel environment was measured after administration of a foot shock in a separate apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18 stainless steel rods (4 mm in diameter), spaced 1.5 cm center to center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5-mA electric shock. Rats were transported in black buckets and shocked immediately upon entry to chambers. After shock, rats were returned to their home cages.

Timeline for behavioral testing

Testing was performed at 1 through 6 months postinjury. A single shock was delivered after neuromotor testing was completed at the 1-month time point.

Immunohistochemistry

Rats were intracardially perfused with 0.9% saline and tissue was collected, then fixed with 4% paraformaldehyde (PFA) overnight at 4°C. Tissue was transferred to a 30% sucrose phosphate-buffered saline solution for 1–2 days, then stored at −80°C. Brains were sectioned at 20 μm and mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) using a cryostat at −22°C. Brain sections were postfixed with 4% PFA for 15 min at room temperature, then treated with 0.3% H_2O_2 for 30 min at room temperature. Immunoreactivity in brain regions associated with anxiety (insula and amygdala) was assessed for markers of microglia (CD11b/c; OX42 labeling) and astrocytes (glial fibrillary acidic protein; GFAP), using an avidin-biotin-horseradish peroxidase (ABC) reaction.[63] Sections were incubated at 4°C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse immunoglobulin G antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400; Vector Laboratories, Burlingame, CA) and reacted with 3',3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). Sections were air-dried overnight and then dehydrated with graded alcohols, cleared in Histoclear and cover-slipped with Permount (Fisher Scientific, Fairlawn, NJ).

Image analysis

Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY), with bright-field illumination at 10× magnification. Densitometric analysis was performed using Scion Image software. Images were analyzed, under blinded conditions, in National Institutes of Health ImageJ (NIH; Bethesda, MD) using grayscale. Signal pixels of a region of interest (ROI) were defined as having gray values 3.5 standard deviations above the mean gray value of a cell-poor area close to the ROI. The number of pixels and the average gray values above the set background were then computed for each ROI and multiplied, giving an integrated densitometric measurement. Six measurements were made for each ROI; measurements were then averaged to obtain a single integrated density value per rat, per region.

Statistical analyses

Results are expressed as mean ± standard error of the mean. Analyses for behavioral measures used analysis of variance (ANOVA) with repeated measures (time after injury) and treatment as the independent variable. The integrated density was measured at one time point (6 months postinjury) and utilized one-way ANOVAs to compare regions between groups. Tukey's honestly significant difference was used to conduct planned pair-wise comparisons to follow up significant overall ANOVAs. Data were analyzed using SPSS® Statistics software (SPSS, Inc., Chicago, IL), and, in all cases, statistical significance was set at $p < 0.05$.

Results

LFPI-induced increases in freezing behavior were observed when rats were placed in a novel context after shock in a separate environment (Fig. 2; $F(3, 20) = 9.029$; $p = 0.001$). Exposed only to this minor additional stressor and before treatment with either MN166 or vehicle, LPFI rats (Fig. 2, white and black bars) froze approximately twice as long as sham-operated rats (Fig. 2, light and dark gray bars) at the 1-month time point: LFPI + vehicle ($p < 0.03$ vs. sham + vehicle and sham + MN166) and LFPI + MN166 ($p < 0.03$ vs. sham + vehicle and sham + MN166), whereas both LFPI groups (before treatment or vehicle) did not differ ($p = 0.94$) statistically.

At 2 months postinjury, after treatment with MN166 or vehicle, freezing in both sham-operated groups remained constant at approximately 25%. There was a drastic reduction (25%) in freezing behavior in MN166-treated rats, with untreated LFPI + vehicle rats freezing approximately 20% more than LFPI + MN166 rats. Freezing differences between sham + vehicle and sham + MN166 control groups and LFPI + MN166-treated rats no longer reached significance ($p = 0.49$ and $p = 0.26$, respectively). Freezing behavior in vehicle-injected LFPI rats remained consistently higher than sham controls ($p < 0.03$ vs. sham + vehicle and sham + MN166), with untreated rats freezing approximately 30% more than sham-operated controls at 2 months postinjury.

At 3 through 6 months postinjury, freezing averages for sham + MN166 and sham + vehicle control groups again remained constant (20 and 25%, respectively). Freezing behavior in vehicle-injected LFPI rats remained consistently higher than drug-treated controls at all post-treatment time points (3 through 6 months; $p < 0.03$ vs. sham + MN166). Freezing behavior in untreated LFPI rats remained higher than sham + vehicle controls at the 3- and 5-month time points postinjury (3 months, $p = 0.00$; 4 months, $p = 0.10$; 5 months, $p = 0.04$; 6 months, $p = 0.13$).

The behavior of MN166-treated LFPI rats remained indistinguishable from controls. Remarkably, freezing differences between sham + MN166- and sham + vehicle-injected control groups and LFPI + MN166-treated rats did not reach significance at any of the post-treatment time points (3 through 6 months; $p > 0.30$ vs. sham + vehicle and sham + MN166). In contrast, untreated LFPI + vehicle-injected rats froze significantly more than treated rats, approximately twice that of the treated rats across all post-treatment time points (3 through 6 months; $p < 0.03$ vs. LFPI + MN166).

The behavioral effects of surgery alone, independent of LFPI, were observed in the sham + vehicle group (Fig. 2, dark gray bars). This group froze more than the sham + MN166-treated group across most time points, in spite of having no significant differences in freezing behavior at baseline. Additionally, whereas the LFPI + vehicle group did freeze more than the sham + vehicle group across the entire study, they did not statistically differ at the 4- and 6-month time points, which may reflect behavioral, immunological, and morphological damage noted by other researchers in response to craniotomy alone.[64]

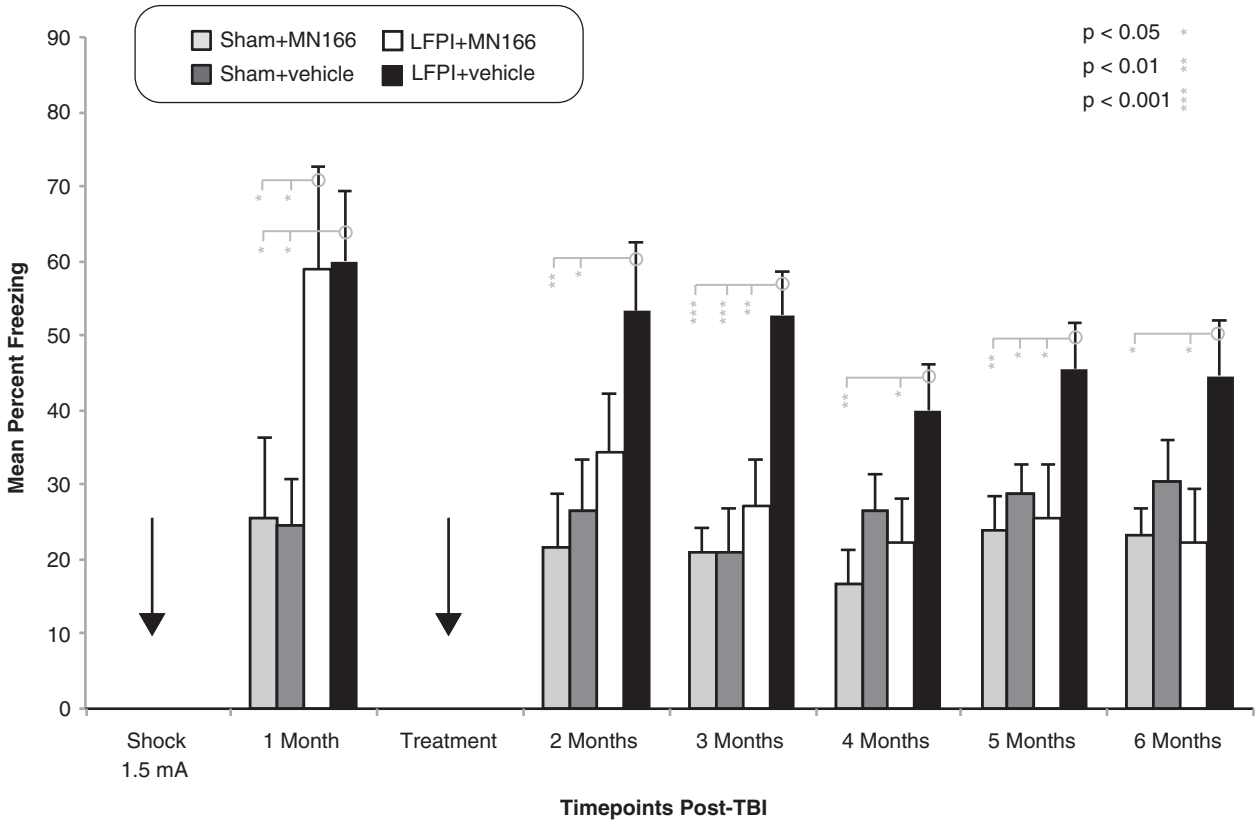

FIG. 2. Freezing behavior in novel context. Sham-operated rats froze approximately 25% before treatment with MN166 or vehicle, whereas LFPI rats froze at significantly higher rates (~60%), indicating greater anxiety-like behavior. After treatment, anxiety-like freezing behavior in LFPI + MN166 rats was reduced, (~25%), compared to LFPI + vehicle rats (~50%). This effect was significant at 3 months and remained through 6 months postinjury. Freezing in sham + MN166 and sham + vehicle rats could not be distinguished from LFPI + MN166-treated rats at all time points after treatment, whereas LFPI + vehicle-injected rats froze significantly more than both sham groups at all post-treatment time points, with the exception of the sham vehicle group at the 4- and 6-month time points. Data represent mean ± standard error of the mean. LFPI, lateral fluid percussion injury; TBI traumatic brain injury.

Disruption of blood vessels and nerves along the scalp suture, mechanical pressure of the drill, and atmospheric exposure when the bone flap is removed can alter vascular physiology and lead to structural and functional impairments after surgery alone.[64,65] Though traditional sham control groups are the standard in TBI research, these results suggest that injury resulting from surgery may increase freezing behavior, although sham + MN166-treated rats showed reductions in freezing behavior from baseline, which does indicate efficacy of MN166 treatment in reducing behavioral effects resulting from craniotomy alone. However, based on these results, future studies will need to include naïve or anesthesia-only controls to determine whether MN166 treatment can reduce anxiety-like freezing behavior to levels of uninjured controls.

LFPI-induced freezing responses were not influenced by motor, vestibular, or locomotive impairments, because neuromotor composite scores of the brain-injured groups (LFPI + MN166 and LFPI + vehicle) did not significantly differ from controls ($F(3, 20) = 0.383$; $p = 0.766$). Rats in all groups consistently received normal scores on fore- and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular, or locomotive functioning as a result of TBI. There were also no significant between-group differences in limb-use asymmetry observed for contra- ($F(3, 20) = 0.058$; $p = 0.981$) and ipsilateral ($F(3, 20) = 0.285$; $p = 0.836$) forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias resulting from injury (Fig. 3A). No significant between-group differences were found in locomotor performance evidenced by distance traveled during the running wheel activity ($F(3, 20) = 0.152$; $p = 0.464$), revealing no postinjury impairments in locomotion (Fig. 3B).

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation, respectively) was assessed in the insula and amygdala in MN166- and vehicle-injected LFPI rats for comparison to sham-operated controls. Representative images (20×), showing GFAP immunoreactivity in several of these regions, are shown in Figure 4, revealing normal astrocyte morphology in both MN166- and vehicle-injected sham controls. LFPI + vehicle rats showed clear signs of reactive astrocytes (Fig. 4; bottom row), whereas LFPI rats treated with MN166 (Fig. 4, third row) were difficult to differentiate from sham-operated control groups.

Immunohistochemistry (IHC) was conducted to assess TBI-induced increases in gliosis and efficacy of MN166 in reducing reactive gliosis in brain regions associated with anxiety. Results revealed increased GFAP labeling in both brain regions examined, confirming that astroglial activation was significantly greater in LFPI + vehicle, compared to other, groups in insula (Fig. 5A, left

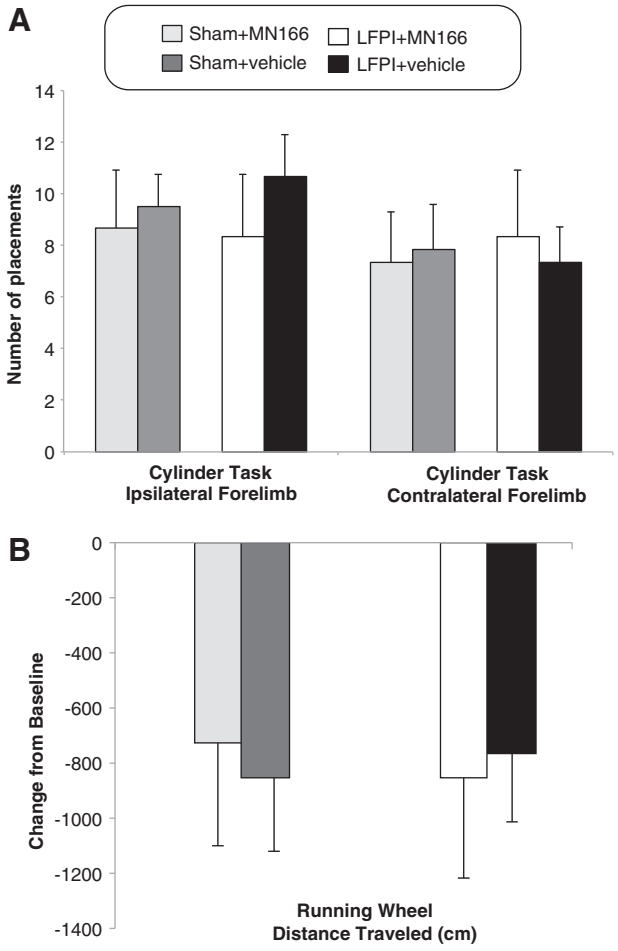

FIG. 3. Cylinder task and running wheel activity at 1 month postinjury. (**A**) LFPI rats mean number of spontaneous forelimb placements (ipsi- and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 month postinjury, indicating no deficits in limb use resulting from injury. (**B**) LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 month postinjury, indicating no impairments in locomotion resulting from injury. Data represent mean ± standard error of the mean. LFPI, lateral fluid percussion injury.

graph; $F(3, 140) = 3.761$; $p = 0.012$) and amygdala (Fig. 5B, left graph; $F(3, 140) = 6.025$; $p = 0.001$). In contrast, no differences in GFAP labeling were observed between sham-operated and LFPI + MN166 groups in either region (insula, $p > 0.60$ vs. sham + vehicle and sham + MN166; amygdala, $p > 0.40$ vs. sham + vehicle and sham + MN166). Whereas MN166-treated LFPI rats were not distinguishable from sham-operated controls, post-hoc analyses revealed that LFPI + vehicle rats had significantly greater astrocytic activation in both brain regions, as compared to controls (Fig. 5A,B, left graphs: insula, $p < 0.02$ vs. LFPI + MN166, sham + vehicle, and sham + MN166; amygdala, $p < 0.01$ vs. LFPI + MN166, sham + vehicle, and sham + MN166).

Analysis of GFAP immunoreactivity in subregions of the insula (Fig. 5A, right graph) also revealed that LFPI + vehicle rats had increased GFAP labeling in agranular ($F(3, 140) = 2.493$; $p = 0.063$), dysgranular ($F(3, 140) = 7.388$; $p = 0.000$), and granular ($F(3, 140) = 2.998$; $p = 0.033$) insular regions. No significant differences between sham-operated and LFPI + MN166 groups were found in subregions of the insula (agranular, $p > 0.70$ vs. sham + vehicle and sham + MN166; dysgranular, $p > 0.20$ vs. sham + vehicle and sham + MN166; granular, $p > 0.20$ vs. sham + vehicle and sham + MN166). Untreated, LFPI + vehicle rats had greater astrocytic activation in all three subregions, as compared to controls (Fig. 5A, right graph: agranular, $p < 0.03$ vs. LFPI + MN166 and sham + vehicle and $p = 0.079$ vs. sham + MN166; dysgranular, $p < 0.01$ vs. LFPI + MN166, sham + vehicle, and sham + MN166; granular, $p = 0.124$ vs. LFPI + MN166, $p = 0.003$ vs. sham + vehicle, and $p = 0.087$ vs. sham + MN166).

In the subregions of the amygdala (Fig. 5B, right graph), GFAP labeling in LFPI + vehicle rats was significantly increased in basolateral amagdala (BLA; $F(3, 140) = 39.154$; $p = 0.000$) and central amygdala (CE; $F(3, 140) = 12.073$; $p = 0.000$) nuclei, compared to controls. Post-hoc analyses revealed that LFPI + vehicle rats had significantly greater astrocytic activation in both subregions (CE, $p < 0.001$ vs. LFPI + MN166, sham + vehicle, and sham + MN166; BLA, $p = 0.001$ vs. LFPI + MN166, sham + vehicle, and sham + MN166). MN166-treated LFPI rats had significantly less GFAP expression than sham + vehicle controls in CE ($p = 0.03$), but did not differ from sham + MN166-treated rats ($p = 0.58$). LFPI + MN166-treated rats also did not differ from sham controls in the BLA ($p = 0.58$ vs. sham + vehicle and $p = 0.06$ vs. sham + MN166).

LFPI + vehicle rats also showed significantly increased microglial activation, as measured by OX-42 labeling, compared to control groups (Fig. 5C), but this was restricted to subregions of the amygdala, such as the CE ($F(3, 140) = 9.290$; $p = 0.000$), and also approached significance in BLA ($F(3, 140) = 2.399$; $p = 0.071$) nuclei. Post-hoc analysis revealed significant increases in microglial activation for LFPI + vehicle rats in CE ($p < 0.001$ vs. LFPI + MN166, sham + vehicle, and sham + MN166) and BLA ($p = 0.009$ vs. sham + MN166). No differences in OX-42 labeling were observed between sham-operated and LFPI + MN166 groups in amygdala, nor were any significant between-group differences found in OX-42 expression for the insula.

Discussion

LFPI-induced anxiety-like behaviors are found at long-term, postinjury time points in untreated brain-injured rats, as compared to those treated with MN166. Pharmacological suppression of immune responses at 1 month postinjury, when anxiety-like behavior has fully developed, markedly reduces long-term behavioral and immunological impairments after TBI (out to 6 months) and restores MN166-treated rats to levels indistinguishable from sham-operated controls. These findings not only implicate chronic neuroinflammation in the development of anxiety-like behaviors after TBI, but also show that delayed treatment is capable of reversing established post-traumatic anxiety behaviors. To our knowledge, this is the first study to examine delayed immunosuppression at long-term injury time points, because other immunosuppressive treatments targeting anxiety-like behaviors have been administered before or within hours of injury.[34,37,38,66–69] These results indicate that the persistence of post-traumatic anxiety may reflect chronic neuroinflammatory neuropathy, a possibility supported by the observation of activated microglia and astrocytes, key cells mediating inflammatory processes, many years after injury in long-term survivors of TBI.[9–12]

Chronic post-traumatic neuroinflammation suggests the presence of a self-perpetuating positive feedback loop, possibly involving reactivation and further promotion of inflammatory mediators after injury in an inflammation-damage-inflammation

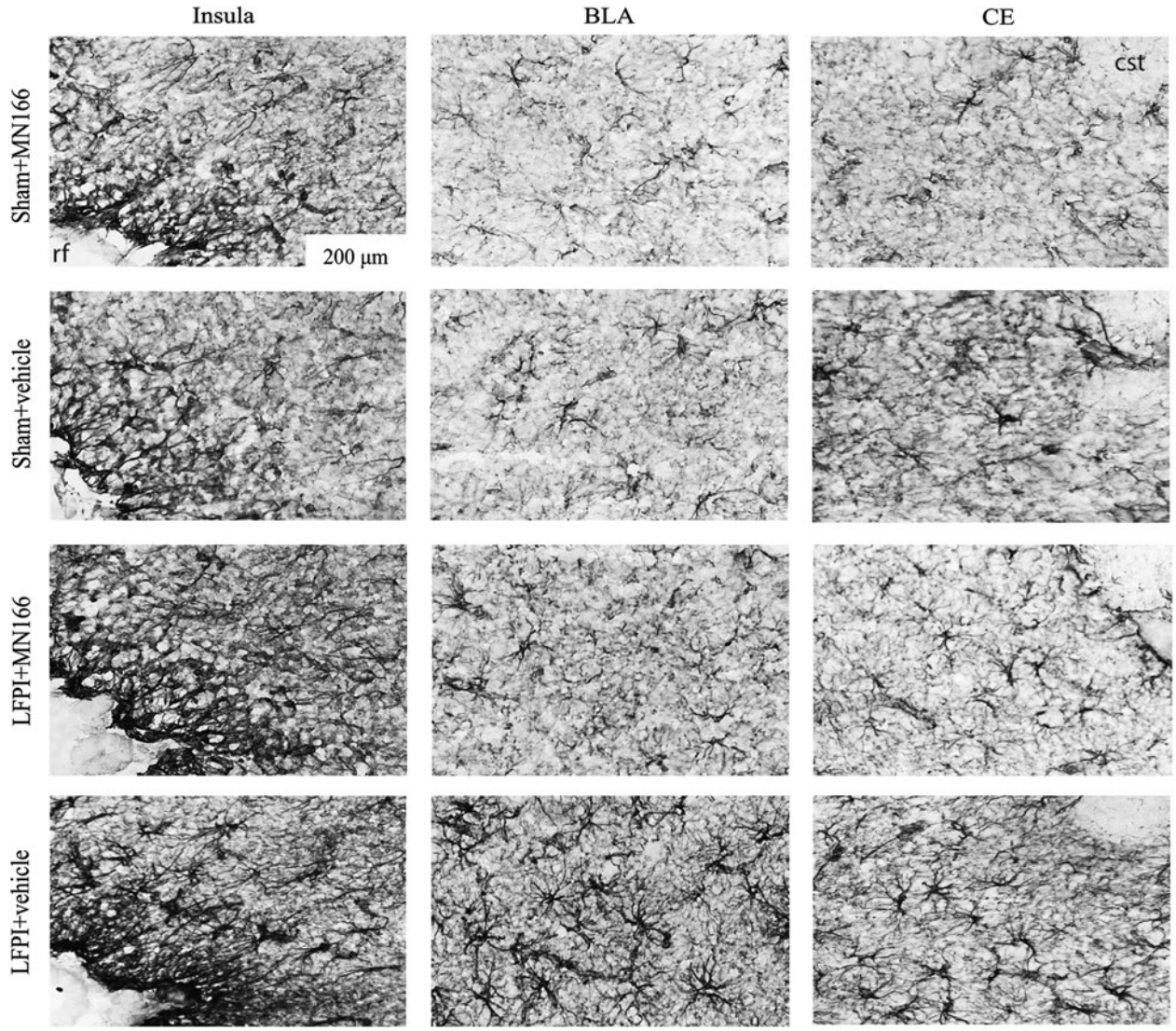

FIG. 4. Representative photomicrographs (20×) depicting GFAP immunoreactivity assessed in the insula and amygdala at 6 months postinjury. Vehicle-injected LFPI rats showed clear signs of reactive astrocytes (bottom row), whereas astrocytes from sham-operated rat tissue appeared to have normal morphology (top rows). LFPI rats treated with MN166 (third row) were difficult to differentiate from sham-operated groups. Rhinal fissure (rf) and commissural stria terminalis (cst). GFAP, glial fibrillary acidic protein; LFPI, lateral fluid percussion injury.

cycle.[70] Stressed, damaged, and injured cells release endogenous danger signals, which trigger local inflammatory responses needed for tissue repair and regeneration.[21,71–73] Damage-associated molecular patterns (DAMPs) play an important role in the propagation of the proinflammatory cascade of innate immunity, promoting the release of cytokines and other inflammatory mediators.[70,74] DAMPs initiate the innate immune response through the activation of APCs, which up-regulate costimulatory and major histocompatibility complex molecules.[21,71,75] APCs respond to endogenous signals through Toll-like receptors (TLRs), which recognize a variety of DAMPs and act as pattern recognition receptors for endogenous molecules.

Microglia are the resident immunological cells and primary APCs of the CNS, remaining quiescent until activated through TLR engagement with DAMPs to perform effector inflammatory and APC functions.[76] Microglial cells contribute to neuroinflammation in response to DAMPS by secreting proinflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α), which amplify the inflammatory response by initiating the production of other cytokines and promoting microglial proliferation and activation of astrocytes.[70] The early phase of TBI-induced reactive gliosis has been reported to begin in with predominant microglia activation that peaks within 1 week,[28,77–82] but continues for several weeks and overlaps later with persistent astrocytic activation.[28,82,83]

Our IHC results support this time course, indicating injury-induced astrogliosis in the insula and amygdala at 6 months postinjury, but less activation of microglia (only significant in the amygdala), suggesting that microglial activation may precede astrocytic activation and modulate the onset and maintenance of astrogliosis.[27,84–88] Lower levels of microglia expression could be the result of assessment at 6 months postinjury, when microglia may have returned to a quiescent or surveying state,[28,89] whereas astrocytic activation persists in a long-lasting, self-perpetuating

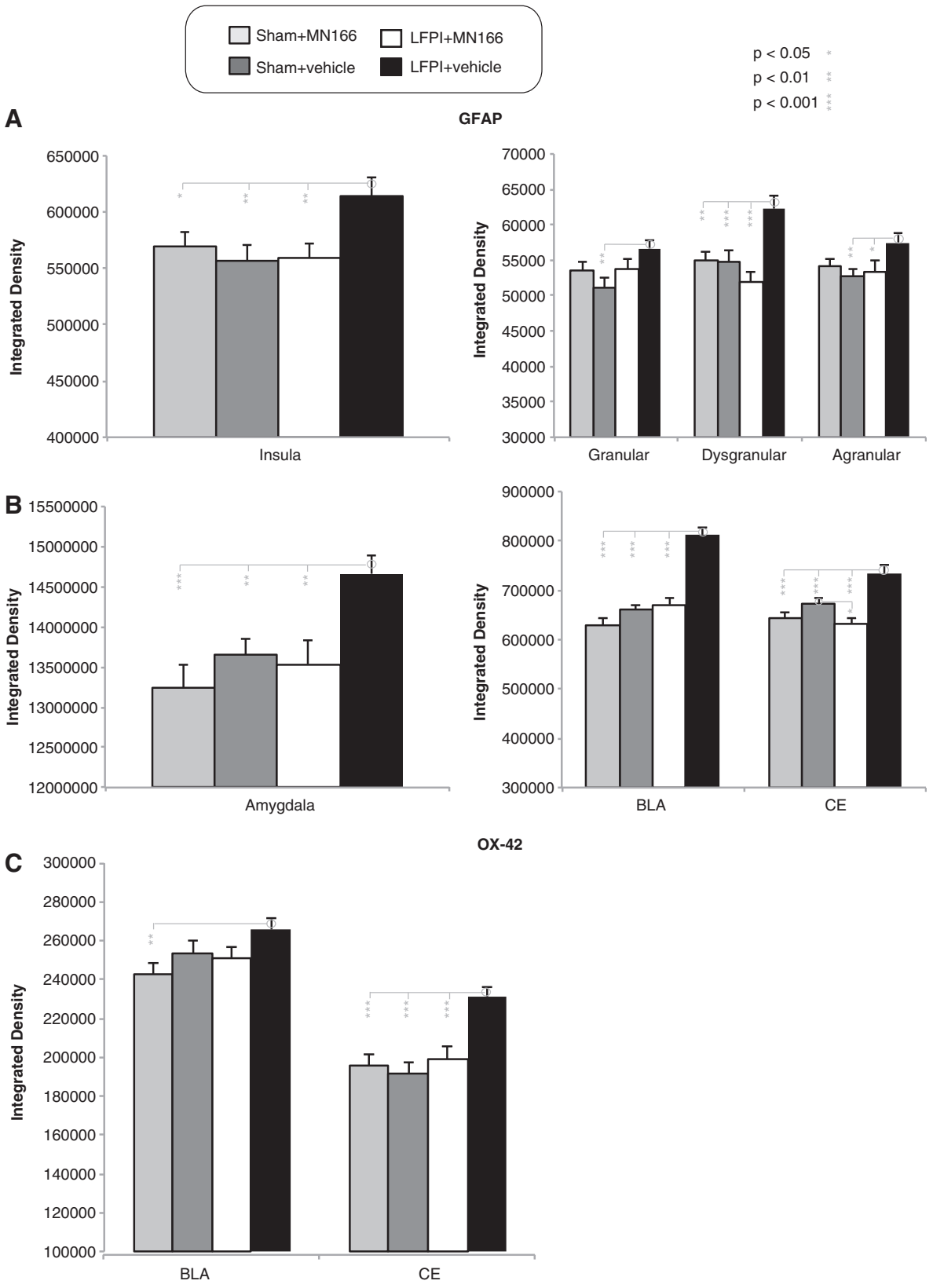

FIG. 5. Astro- and microglial activation in insula and amygdala at 6 months postinjury. LFPI + vehicle rats had increased reactive gliosis postinjury, as evidenced by increased glial activation, compared to controls, and treatment with MN166 reduced gliosis levels in LFPI rats to those of sham-operated controls. (**A** and **B**) LFPI + vehicle rats had significantly increased in GFAP labeling in both regions, indicating higher astroglial activation, compared to sham-operated and LFPI + MN166-treated rats. (**C**) In the CE, microglial activation was greater in LFPI + vehicle-injected rats, compared to both sham-operated groups and LFPI + MN166-treated rats, and was approaching significance in BLA. Data represent mean ± standard error of the mean. LFPI, lateral fluid percussion injury; GFAP, glial fibrillary acidic protein; CE, central amagdala; BLA, basolateral amygdala.

inflammatory response in the brain that exceeds early neuroprotection and results in neurodegenerative changes capable of continuing the inflammatory cycle.[9,27,90]

Chronic inflammation has been observed in a number of studies examining patients with trauma-related anxiety disorders, reporting increases in downstream mediators, such as peripheral elevations of TNF-α, interferon-gamma (IFN-γ), IL-1β, and IL-6, in patients with PTSD,[13–16] elevations of TNF-α and IL-6 in patients with OCD,[17] and elevations in proinflammatory cytokines and chemokines (monocyte chemoattractant protein 1, macrophage inflammatory protein 1 alpha, IL-1α, IL-1β, IL-6, IL-8, Eotaxin, granulocyte macrophage colony-stimulating factor, and IFN-γ) in individuals with panic disorder and PTSD.[18] Despite compelling evidence implicating excessive inflammatory actions and a generalized inflammatory state in the development of anxiety disorders after TBI, central measures of proinflammatory cytokine elevations specifically related to human PTSD and other anxiety disorders have not yet been performed. However, the current results provide evidence for chronic neuroinflammation in the development and maintenance of post-traumatic anxiety in an animal model, as indicated by elevated immunoreactivity in the amygdala and insula at 6 months postinjury.

The amygdala and insula have long been associated with human anxiety disorders. Studies in patients with PTSD implicate exaggerated responses of the amygdala and insula,[91–95] impaired inhibition of medial prefrontal cortex and anterior cingulate,[94,96–98] and decreased hippocampal volume.[97,99,100] Other neuroimaging reports of patients with non-trauma-related OCD and phobia, as well as those with PTSD, have revealed that aversive anticipation (a hallmark of anxiety) involves increased activation of both the amygdala and insula.[92] Evidence for the specific involvement of these brain areas in human post-traumatic anxiety is complemented by animal models, including findings of increased PTSD-related traits and increased Stathmin 1 (a protein known to increase fear responses) expression in the amygdala after blast injury,[101] increased fear conditioning and up-regulation of excitatory N-methyl-D-aspartate receptors (crucial receptors for normal fear learning and memory) in the amygdala after concussive injury,[33] and our current results showing increased anxiety-like behavior and reactive gliosis in insula and amygdala at long-term time points after LFPI. However, though these results support findings from our previous study[34] indicating increased gliosis in these areas, the insula and amygdala are not the only regions involved in anxiety and do not exclude the possibility that other regions may be contributing to the results reported here. Future studies will be aimed at characterizing the model and inclusion of other brain regions involved in human anxiety, including cingulate cortex, hippocampus, medial prefrontal cortex, locus coeruleus, and the bed nucleus of the stria terminalis.[94,97,99,102,103]

Although the exact role of the immune system in the pathogenesis of anxiety disorders after TBI remains unknown, neuroinflammation is emerging as a potential target. The present findings of treatment-related reductions in anxiety-like behaviors and reactive gliosis in brain regions associated with anxiety support the use of immunosuppression to improve functional outcome after TBI. Peri-injury and immediate postinjury immunosuppression have been found to be neuroprotective after TBI in rodents, resulting in increased structural preservation and improved functional outcomes.[104] Early administration of the immunosuppressant drugs, minocycline, statins, cyclosporin A, and FK506, have been shown to exert anti-inflammatory effects through suppression of micro- and astroglial production of IL-1β, TNF-α, and IL-6, resulting in reduced functional deficits, cerebral edema, and brain lesion volumes,[35–38,66] improving mitochondrial preservation, reducing dendritic spine loss, and improving cognitive performance and functional motor recovery.[105,106] Our previous investigation found that peri-injury Ibudilast treatment attenuated glial cell activation at the time of injury, resulting in reduced anxiety-like behaviors and immunological impairments after LFPI. [34]

These immunosuppressant drugs all have direct inhibitory effects on microglia and astrocytes, leading to better functional recovery after TBI; however, these treatments require rapid administration and reduce the therapeutic window to the day of injury. The current work shows reversal of established anxiety-like behaviors and reactive gliosis at 1 month postinjury, delayed treatment time points that have not been tested with any other immunosuppressive interventions, in spite of substantial evidence that many molecular, biochemical, and immunological changes occur for many months after injury and that clinical intervention may not be possible at early stages of TBI. Ongoing inflammation represents a window of opportunity for therapeutic intervention to prevent progressive tissue damage, loss of function, and possibly interrupt the progression of neuropathological conditions after injury.

Our finding that delayed immunosuppression is capable of reversing established post-traumatic anxiety behaviors and immunological impairments through 6 months after TBI contributes to growing evidence that the critical window for treatment after TBI can be expanded to include those suffering from long-term TBI-related disabilities. Studies have shown that delayed treatment (24 h) with erythropoietin, a novel neuroprotective cytokine found to improve neuronal survival through attenuation of cytokine production and inflammation, improved sensorimotor functional recovery, reduced hippocampal cell loss, enhanced neurogenesis, and improved neurological outcomes after controlled cortical impact (CCI) and weight-drop rodent TBI models.[107,108] Similarly, a recent study reported reduced chronic inflammation and neurodegeneration after activation of metabotropic glutamate receptor 5 with the specific agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), which has been shown to decrease microglial activation and release associated proinflammatory mediators. The study delayed treatment until 1 month after CCI in mice, delivering a single intracerebroventricular injection of CHPG. The results revealed reductions in reactive gliosis, hippocampal cell loss, reduced lesion progression, and improved motor and cognitive recovery, compared to untreated controls.[109]

Immunosuppression of chronic neuroinflammation may hold promise as a therapeutic target in treatment of established anxiety disorders after TBI. It has been shown here that inflammation produced by neuroimmune responses after injury plays a role in TBI-induced anxiety, and delayed immunosuppression leads to better functional outcomes at long-term postinjury treatment points after TBI. Delayed, postinjury suppression of glial cell activation could therefore expand the clinical window for treatment of TBI-induced anxiety disorders in humans.

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Author Disclosure Statement

No competing financial interests exist.

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Acute Neuroimmune Modulation Attenuates the Development of Anxiety-Like Freezing Behavior in an Animal Model of Traumatic Brain Injury

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Abstract

Chronic anxiety is a common and debilitating result of traumatic brain injury (TBI) in humans. While little is known about the neural mechanisms of this disorder, inflammation resulting from activation of the brain's immune response to insult has been implicated in both human post-traumatic anxiety and in recently developed animal models. In this study, we used a lateral fluid percussion injury (LFPI) model of TBI in the rat and examined freezing behavior as a measure of post-traumatic anxiety. We found that LFPI produced anxiety-like freezing behavior accompanied by increased reactive gliosis (reflecting neuroimmune inflammatory responses) in key brain structures associated with anxiety: the amygdala, insula, and hippocampus. Acute peri-injury administration of ibudilast (MN166), a glial cell activation inhibitor, suppressed both reactive gliosis and freezing behavior, and continued neuroprotective effects were apparent several months post-injury. These results support the conclusion that inflammation produced by neuroimmune responses to TBI play a role in post-traumatic anxiety, and that acute suppression of injury-induced glial cell activation may have promise for the prevention of post-traumatic anxiety in humans.

Key words: lateral fluid percussion injury; neuroinflammation; post-traumatic stress disorder; traumatic brain injury

Introduction

The long-term consequences of traumatic brain injury (TBI) include a heightened risk of neuropsychiatric disorders, of which anxiety disorders are the most prevalent (Moore et al., 2006; Rao and Lyketsos, 2000; Vaishnavi et al., 2009). Studies of the etiology of anxiety disorders implicate exaggerated responses of the amygdala and insula (Carlson et al., 2011; Rauch et al., 1997; Shin and Liberzon, 2010; Simmons et al., 2006; Stein et al., 2007), impaired inhibition of the medial prefrontal cortex and anterior cingulate (Davidson, 2002; Milad et al., 2009; Shin and Liberzon, 2010; Shin et al., 2006), and decreased hippocampal volume (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Yet, whether and how TBI induces neurochemical, structural, and functional abnormalities in these structures is poorly understood.

There is increasing evidence that excessive inflammatory actions of the neuroimmune system may contribute to the development of anxiety disorders following TBI (Gasque et al., 2000; Hoge et al., 2009; Shiozaki et al., 2005; Spivak et al., 1997; Tucker et al., 2004; von Känel et al., 2007). Microglial cells are the first line of defense and primary immune effector cells in the central nervous system (CNS), and respond immediately to even small pathological changes from damaged cells, producing proinflammatory cytokines and toxic molecules that compromise neuronal survival (Aloisi, 2001; Gehrmann, 1996; Gonzalez-Scarano and Baltuch, 1999; Town et al., 2005). This rapid microglial response often precedes the more delayed, yet prolonged activation of astrocytes, and is thought to be involved with the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; Hanisch, 2002; Herber et al., 2006; Iravani et al., 2005; McCann et al., 1996; Zhang et al., 2010). It is well established that microglia and astrocytes are activated during the innate immune response to brain injury, leading to the expression of high levels of proinflammatory cytokines, most notably interleukin-1β

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(IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). While glial activation is typically neuroprotective (Aloisi, 2001; Farina et al., 2007), the chronic inflammatory responses and exaggerated proinflammatory cytokine levels observed following injury initiate neurotoxic processes resulting in secondary tissue damage (Gasque et al., 2000; Hailer, 2008; Lehnardt, 2010; Simi et al., 2007), neuronal death (Beattie et al., 2010; Brown and Bal-Price, 2003; Schmidt et al., 2005; Sternberg, 1997), secondary injury cascades (Ansari et al., 2008a,2008b; Bains and Shaw, 1997; Cernak et al., 2001b, 2001a), and neuronal hyperexcitability (Beattie et al., 2010; Hailer, 2008; Riazi et al., 2008; Rodgers et al., 2009), all of which may contribute to the dysfunction of brain regions associated with anxiety.

Recently developed animal models of post-traumatic anxiety (Baratz et al., 2010; Fromm et al., 2004; O'Connor et al., 2003; Jones et al., 2008; Liu et al., 2010; Sönmez et al., 2007; Vink et al., 2003; Wagner et al., 2007) permit examination of the possible contributions of brain inflammation. Tests of post-traumatic anxiety in these models have typically included standard measurements of exploratory preference in mildly stressful environments, such as an open-field or elevated-plus testing apparatus. However, it has been frequently noted that measures of exploratory preference may be confounded by a marked overall decrease in exploration in brain-injured animals (Fromm et al., 2004; O'Connor et al., 2003; Vink et al., 2003). Decreased exploration cannot be attributed to TBI-induced motor deficits since numerous studies report only transient ($\sim$ 1 week) deficits following trauma (Baratz et al., 2010; Bouilleret et al., 2009; Cutler et al., 2005,2006a,2006b; Dixon et al., 1996; Fassbender et al., 2000; Frey et al., 2009; Goss et al., 2003; Kline et al., 2007; Liu et al., 2010; Taupin et al., 1993; Wagner et al., 2007; Yan et al., 1992). Rather, TBI-induced decreases in exploration have been attributed to the indirect effects of freezing (a primary component of the rodent's natural defensive behavior repertoire; Blanchard and Blanchard, 1988), suggesting an abnormally heightened response to stress in brain-injured rats (O'Connor et al., 2003; Fromm et al., 2004; Vink et al., 2003).

Based on these results, we tested the hypothesis that trauma-induced innate immune responses contribute to the development of anxiety-like behaviors in rats by directly examining freezing responses to a minor (novel environment), and major (foot-shock) stressor, following lateral fluid percussion injury (LFPI; a clinically-relevant animal model of human closed-head injury). We also tested the effectiveness of a glial cell activation inhibitor, ibudilast (MN166), in attenuating post-injury freezing behavior and reducing reactive gliosis in brain regions associated with hyperexcitability in anxiety disorders.

Methods

Sixty adult virus-free male Sprague-Dawley rats (275–325 g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature-controlled (23 $\pm$ 3°C) and light-controlled (12-h:12-h light:dark cycle) rooms with *ad libitum* access to food and water. All procedures were performed in accordance with the University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. The rats were randomly assigned to 1 of 10 groups (n = 6/group). Six groups (surgically-naïve, sham-operated, sham-operated + vehicle, sham-operated + MN166, LFPI + vehicle, and LFPI + MN166) were shocked immediately after behavioral testing at 1 month post-surgery (sham surgery or LFPI in the experimental rats). Surgically-naïve rats received no injections or surgery, whereas sham-operated rats received surgery but were not injected. The final four groups received sham or LFPI surgery and either vehicle injections or MN166 treatment. Another four groups (sham-operated + vehicle, sham-operated + MN166, LFPI + vehicle, and LFPI + MN166) were run separately in a sucrose preference test to assess anhedonia (the inability to experience pleasure, a core symptom of human depression), without exposure to stressors (anxiety tests and foot shock).

Lateral fluid percussion injury

LFPI rats were anesthetized with halothane (4% induction, 2.0–2.5% maintenance) and mounted in a stereotaxic frame. The LFPI used in this study has been described previously (Frey et al., 2009; McIntosh et al., 1989; Thompson et al., 2005). A PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL) was used to deliver standardized pressure pulses of air to a standing column of fluid. A 3.0-mm-diameter craniotomy was centered 3 mm caudal to the bregma and 4.0 mm lateral to the sagittal suture, with the exposed dura remaining intact. A female Luer-Lok hub (inside diameter 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. Following hub placement, the animal was removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate impact force (2.0 atmospheres; 10 msec). The injury cap was then removed, the scalp was sutured, and the rats were returned to their home cages for recovery. Sham-operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration

MN166 (MediciNova Inc., San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation, which has been found to reduce glia-induced neuronal death through the suppression of nitric oxide, reactive oxygen species, and proinflammatory mediators (Mizuno et al., 2004; Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg, 1 mL/kg subcutaneously in corn oil) 24 h prior to LFPI, the day of surgery and LFPI, and 3 days following LFPI. Weight was recorded prior to each dosing, and treatment was administered at the same time each day to maintain constant levels across a 24-h period. Dose selection was based on prior animal pharmacology results (Ledeboer et al., 2007), demonstrating MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high-dose regimens in clinical development. MN166 administered using this regimen yields plasma and CNS concentrations that are linked to molecular target actions, including most potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010), and secondarily, phosphodiesterase (PDE)-4 and PDE-10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (Wang et al., 2011). Such

dosing regimens have clearly been linked to glial attenuation in other animal models (Ledeboer et al., 2007), and the anti-inflammatory actions of MN166 have recently been shown to suppress cerebral aneurysms in a dose-dependent manner (Yagi et al., 2010).

Tests of motor, vestibular, and locomotive performance

Baseline testing of motor, vestibular, and locomotive performance in all groups was conducted immediately prior to surgery, and again following a 1-week recovery period. These tests included ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use, and limb preference (Bland et al., 2000,2001), toe spread to assess gross motor response (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000; Woodlee et al., 2005), a catalepsy rod test to assess postural support and mobility (Sanberg et al., 1988), bracing to assess postural stability and catalepsy (Morrissey et al., 1989;Schallert et al., 1979), and air righting to assess dynamic vestibular function (Pellis et al., 1991a,1991b). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The individual test scores were summed and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following CNS injury in rats (Schallert et al., 2000,2006), and post-injury locomotor activity was assessed through distance traveled on a running wheel. Both tasks were scored for 5 min under red light ($\sim$ 90 lux).

Behavioral measures

A novel environment was used to assess freezing behavior in response to a minor stressor (Dellu et al., 1996). The environment consisted of a standard rat cage with one vertically-striped and one horizontally-striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. The rats were tested (5 min), and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement except for heartbeat/respiration, and was recorded in 10-sec intervals.

Freezing behavior in the novel environment was measured before and after administration of a foot shock in a separate shock apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5-mA electric shock. The rats were transported in black buckets and shocked immediately upon entry to the chambers. Following shock, the rats were returned to their home cages.

A sucrose preference test was also performed in separate groups of rats that did not receive foot shock or testing in the novel environment. This task is commonly used to measure anhedonia in rodent models of depression (Monleon et al., 1995; Willner, 1997). The sucrose preference task was included because anxiety and depression share high rates of co-morbidity in humans (Moore et al., 2006), and was assessed as a possible confound to freezing behavior, due to possible co-occurrence of depression-like behavior. The rats were first habituated to sucrose solution, and were tested during the dark phase of the light/dark cycle to avoid the food and water deprivation necessary when testing during the light phase. Day 1 and day 2 consisted of habituation, day 3 and day 4 were baseline (averaged), and day 5 was the first test day. The rats were presented with two pre-weighed bottles containing 2% sucrose solution or tap water for a period of 4 h. Thirty minutes into the task the bottles were swapped to force preference and counter for placement effects. Total sucrose intake and sucrose preference, calculate by: (sucrose intake/(sucrose intake + water intake * 100), were measured.

Timeline for behavioral testing

Following a 2-week recovery period from sham operation or LFPI in experimental animals, all groups except those to be evaluated for sucrose preference were tested in the novel context. Testing was performed at 2 weeks, and 1, 2, and 3 months post-surgery. Shock was delivered after behavioral testing was completed at the 1-month time point. Tests for sucrose preference were performed at 2 weeks, 1 month, and 3 months post-surgery, with no intervening foot shock.

Immunohistochemistry

Immunoreactivity for OX-42 (targets CD11b/c, a marker of microglial activation), and glial fibrillary acidic protein (GFAP; a marker of astrocyte activation) were measured using an avidin-biotin-horseradish peroxidase (ABC) reaction (Loram et al., 2009). Brain sections (12 μm) were cut on a cryostat and mounted onto poly-L-lysine-coated slides and stored at -80°C. Sections were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H_2O_2 for 30 min at room temperature. The sections were incubated at 4°C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA), or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, the sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). The sections were washed and incubated for 2 h at room temperature in ABC (1:400; Vector Laboratories, Burlingame, CA), and reacted with 3′,3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Glucose oxidase and β-D-glucose were used to generate hydrogen peroxide. Nickelous ammonium sulfate was added to the DAB solution to optimize the reaction product. The sections were air-dried overnight and then dehydrated with graded alcohols, cleared in Histo-Clear, and cover-slipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometric analysis was performed using Scion Image software.

Image analysis

The slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY), with bright-field illumination at 10$\times$ magnification. The images were opened in ImageJ, converted into gray scale, and rescaled from inches to pixels. Background areas were chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure

(integrated gray level). Four measurements were made for each ROI; the measurements were then averaged to obtain a single integrated density value per rat, per region. All measurements were taken while blind to treatment group.

Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM). Analyses for all behavioral variables used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density from the histology was only conducted at one time point, and utilized one-way ANOVAs to compare regions between groups. Data were analyzed using SPSS software, and in all cases statistical significance was set at $p < 0.05$.

Results

Neuromotor composite scores of the brain-injured groups (LFPI + MN166 and LFPI + vehicle) did not significantly differ from controls [$F_{(3,20)} = 0.803$, $p = 0.508$]. Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular, or locomotive functioning due to TBI. There were also no significant between-group differences in limb-use asymmetry observed for contralateral [$F_{(5,29)} = 0.544$, $p = 0.741$] and ipsilateral [$F_{(5,29)} = 0.428$, $p = 0.826$] forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias due to injury (Fig. 1A). No significant between-group differences were found in locomotor performance as evidenced by distance traveled during the running wheel activity [$F_{(5,29)} = 0.069$, $p = 0.996$], revealing no post-injury impairments in locomotion (Fig. 1B). There were no significant between-group differences in the sucrose preference task [$F_{(3,21)} = 0.338$, $p = 0.798$], indicating no impairments in hedonic states post-injury.

Despite normal motor, vestibular, and locomotive function, LFPI produced large increases in freezing behavior when rats were placed in a novel context [Fig. 2; $F_{(5,30)} = 9.539$, $p < 0.0001$]. Exposed only to this minor stressor (i.e., at 2-week and 1-month post-injury measurements conducted prior to shock), LFPI rats injected with either MN166 or vehicle (Fig. 2, white and black bars, respectively) froze approximately twice as long as naïve or sham-operated rats (Fig. 2; light and dark grey bars, respectively; $p < 0.01$). At the 2- and 3-month measurement time points, following the additional major stressor of shock (arrow in Fig. 2), freezing in both naïve and sham-operated rats remained constant at approximately 10%. Freezing in LFPI rats treated with MN166 remained consistently higher than their controls ($p < 0.001$) but, while appearing higher compared to earlier post-injury measurements in the same animals, this increased freezing compared to naïve and sham-operated rats before (1 month) and following (2 months) shock did not reach significance ($p = 0.316$). By contrast, LFPI + vehicle rats nearly doubled their freezing time, to approximately 50% (Fig. 2, black bars) compared to pre-shock values ($p < 0.001$), freezing approximately twice as long as LFPI + MN166 rats ($p < 0.001$), and five times as long as naïve and sham-operated controls ($p < 0.001$), at the 2- and 3-month post-injury time points.

The behavioral effects of injections alone, independent of LFPI, are reflected in the sham-surgery groups with injections

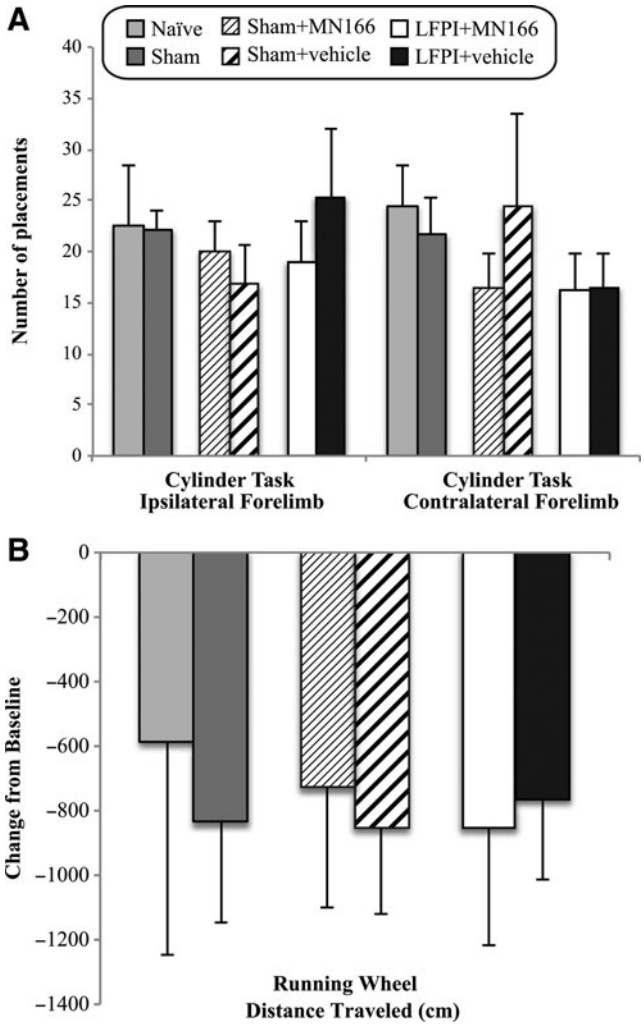

FIG. 1. Cylinder task and running wheel activity at 1 week post-injury. (**A**) For LFPI rats, the mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 week post-injury. A reduction was seen in contralateral limb use in injured rats, but this reduction did not reach significance ($p = 0.741$). (**B**) For LFPI rats, the mean change in distance traveled in the running wheel did not significantly differ from controls at 1 week post-injury. Data represent mean ± standard error of the mean (LFPI, lateral fluid percussion injury).

of either MN166 or vehicle (Fig. 2, narrow and broad diagonal lines, respectively). Sham-operated rats tended to freeze more than un-injected naïve and sham-operated controls, reaching significance for both groups at the 2- and 3-month measurement time points ($p < 0.01$), and suggesting that injections alone are aversive and can contribute to subsequent freezing. However, even at pre-shock measurement points, LFPI animals that received the same injections of MN166 or vehicle froze significantly more than injected controls ($p < 0.01$), indicating substantial enhancement of freezing produced by LFPI. This effect became more apparent following shock, when LFPI + vehicle rats froze twice as long as the injected controls ($p < 0.001$). By contrast, LFPI + MN166 rats were not distinguishable from either injected control group following

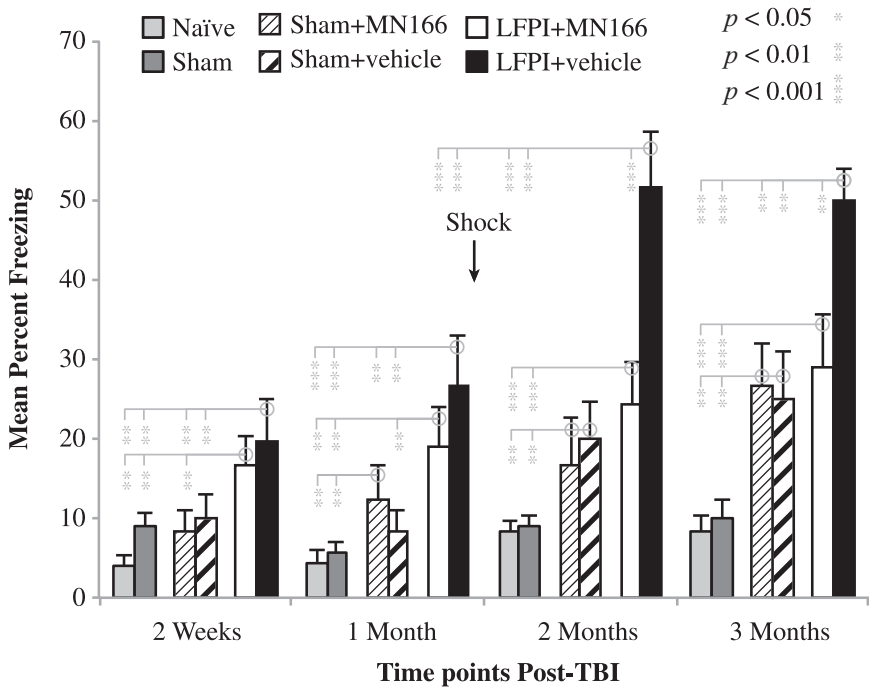

FIG. 2. Freezing behavior in a novel context. Both surgically-naïve and sham-operated rats froze approximately 5–10% at post-surgical measurement points before (2 weeks and 1 month) after (2 and 3 months) foot shock (arrow). In contrast, LFPI rats froze significantly longer (~ 20%) than controls before shock. After shock, untreated LFPI rats (LFPI + vehicle) nearly doubled their freezing time (~ 50%), whereas treated LFPI rats (LFPI + MN166) showed only a slight increase (~25%), that did not reach significance ($p = 0.316$). The effects of injection alone (sham + MN166 and sham + vehicle) were to increase freezing behavior compared to un-injected naïve and sham-operated rats, particularly at the 2- and 3-month post-shock measurement time points, when freezing in these rats could not be distinguished from LFPI rats treated with MN166. Data represent mean ± standard error of the mean (LFPI, lateral fluid percussion injury; TBI, traumatic brain injury).

shock, suggesting that their elevated freezing compared to naïve and sham-operated animals was the result of injections alone, and that MN166 eliminated the exaggerated freezing response to shock characterizing LFPI + vehicle rats.

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation) was assessed in the insula, amygdala, and hippocampus in brain-injured rats for comparison to sham-operated and surgically-naïve rats. Representative images (40×), showing GFAP immunoreactivity in several of these regions, are shown in Figure 3, revealing normal astrocyte morphology in surgically-naïve and sham-operated rats. LFPI + vehicle rats showed clear signs of reactive astrocytes (Fig. 3, bottom row). LFPI rats treated with MN166 (Fig. 3, third row) were difficult to differentiate from sham-operated or surgically-naïve control groups.

Densitometry of GFAP labeling in all areas examined confirmed that activation of astrocytes was significantly greater in LFPI compared to all other groups in the insula [Fig. 4A, left bars; $F_{(3,19)} = 13.17$, $p < 0.0001$], amygdala [Fig. 4B, left bars; $F_{(3,18)} = 7.54$, $p < 0.002$], and hippocampus [Fig. 4C, left bars; $F_{(3,15)} = 8.47$, $p < 0.002$]. In contrast, no differences in GFAP labeling were observed between the surgically-naïve, sham-operated, and LFPI + MN166 groups, in any of the regions examined. While MN166-treated LFPI rats were not distinguishable from surgically-naïve or sham-operated controls, *post-hoc* analyses revealed that LFPI + vehicle rats had significantly greater astrocyte activation in all three brain regions compared to controls (Fig. 4A–C): insula ($p < 0.002$ versus the surgically-naive, sham-operated, and LFPI + MN166 groups), amygdala ($p < 0.02$ versus the surgically-naive, sham-operated, and LFPI + MN166 groups). and hippocampus ($p < 0.03$ versus the surgically-naive, sham-operated and LFPI + MN166 groups).

Analysis of GFAP immunoreactivity in sub-regions of the insula (Fig. 4A, right bars), amygdala (Fig. 4B, right bars), and hippocampus (Fig. 4C, right bars), also revealed no differences between the surgically-naïve, sham-operated, and LFPI + MN166 groups. As in the regional analysis, LFPI + vehicle rats showed increased astrocyte activation over controls in most sub-regions examined. In the insula, LFPI + vehicle rats showed significantly increased GFAP labeling in agranular [$F_{(3,19)} = 16.778$, $p < 0.0001$], dysgranular [$F_{(3,19)} = 6.042$, $p < 0.005$], and granular [$F_{(3,19)} = 5.277$, $p < 0.008$] regions, compared to control groups. In the amygdala, GFAP labeling in LFPI + vehicle rats was significantly increased in the basolateral amygdala (BLA) [$F_{(3,18)} = 4.050$, $p < 0.023$] and central amygdala (CE) [$F_{(3,18)} = 5.012$, $p < 0.011$] nuclei, compared to controls. LFPI + vehicle rats also showed increased GFAP expression in the hippocampus, but this was only significant in CA3 [$F_{(3,18)} = 3.810$, $p < 0.03$], and approached significance in CA1 [$F_{(3,17)} = 3.234$, $p = 0.055$].

LFPI + vehicle rats also showed significantly increased microglia activation compared to control groups as measured by OX-42 labeling, but this was restricted to the insula [Fig. 4D; $F_{(3,19)} = 5.59$, $p < 0.007$]. Analysis of sub-regions of the insula also revealed increases in microglial activation for LFPI + vehicle rats, and *post-hoc* comparisons showed that LFPI alone significantly increased OX-42 labeling in agranular

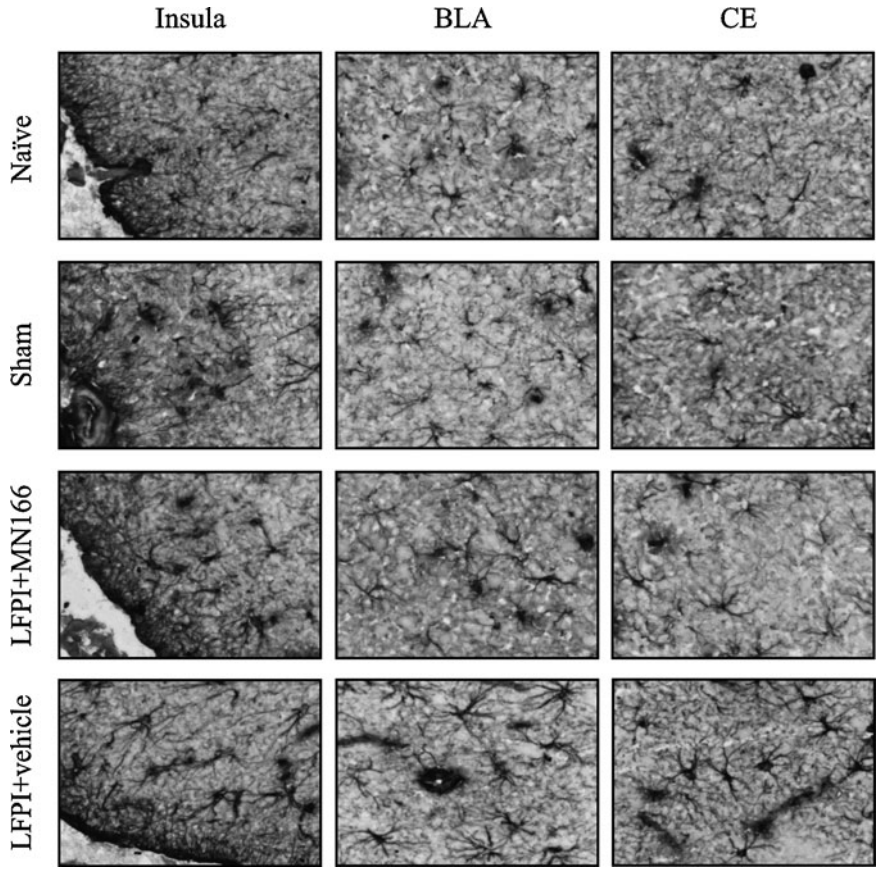

FIG. 3. Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) as assessed in the hippocampus, amygdala, and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham-operated rats appeared to have normal astrocyte morphology. LFPI rats treated with MN166 (third row from top) were difficult to differentiate from surgically-naïve and sham-operated animals (LFPI, lateral fluid percussion injury; GFAP, glial fibrillary acidic protein; BLA, basolateral amygdala; CE, central amygdala).

[$F_{(3,19)} = 11.186$, $p < 0.0001$] and granular [$F_{(3,18)} = 3.740$, $p < 0.03$] areas, and that it approached significance [$F_{(3,19)} = 2.742$, $p < 0.072$] in dysgranular areas. No differences in OX-42 labeling were observed between the surgically-naïve, sham-operated, and LFPI + MN166 groups, in any insular regions examined. No significant between-group differences were found in OX-42 expression for the amygdala or hippocampus.

Discussion

These data suggest a link between injury-induced brain inflammation and post-traumatic anxiety. Rats with LFPI display freezing responses to the minor stress of a novel environment that is 2–3 times normal, and which unlike controls, is nearly doubled by the delivery of a major foot-shock stressor. LFPI also results in marked reactive gliosis in brain regions associated with anxiety. The possibility that post-traumatic brain inflammation and gliosis may contribute to the anxiety-like behavior observed here is supported by the effects of the glial-cell activation inhibitor MN166. MN166 reduces reactive gliosis and TBI-induced freezing behavior, rendering these animals histologically and behaviorally indistinguishable from naïve and sham-operated controls. To our knowledge, this is the first study to report pharmacological immunosuppression resulting in the reduction of anxiety-like behaviors following TBI.

A possible mechanism for neuroimmune-induced post-traumatic anxiety

Our finding of prolonged reactive gliosis in brain structures including, but likely not confined to, the hippocampus, amygdala, and insular cortex, suggests that these structures may contribute to the persistent enhanced freezing of our brain-injured animals in reaction to a novel environment. All three structures have been implicated in rodent research investigating the pathogenesis of anxiety (Canteras et al., 2010; Davidson, 2002; Davis, 1992; Davis et al., 1994; Paulus and Stein, 2006; Rauch et al., 2006; Vyas et al., 2004) and fear behavior in the rat (Liu et al., 2010; Milad et al., 2009; Rosen and Donley, 2006; Sullivan, 2004).

The mechanisms by which immune responses may contribute to dysfunction of these structures remain to be determined. It is well established that LFPI in the rat results in activation of microglia and astrocytes as part of the innate immune response to insult. A number of studies indicate that LFPI-induced reactive gliosis follows a distinct time course, beginning with predominant microglia activation that peaks within a week (Clausen et al., 2009; Grady et al., 2003; Gueorguieva et al., 2008; Hill et al., 1996; Nonaka et al., 1999; Yu et al., 2010), but continues for several weeks and overlaps later with persistent astrocytic activation (D'Ambrosio et al., 2004; Yu et al., 2010). Microglia are resident macrophages and

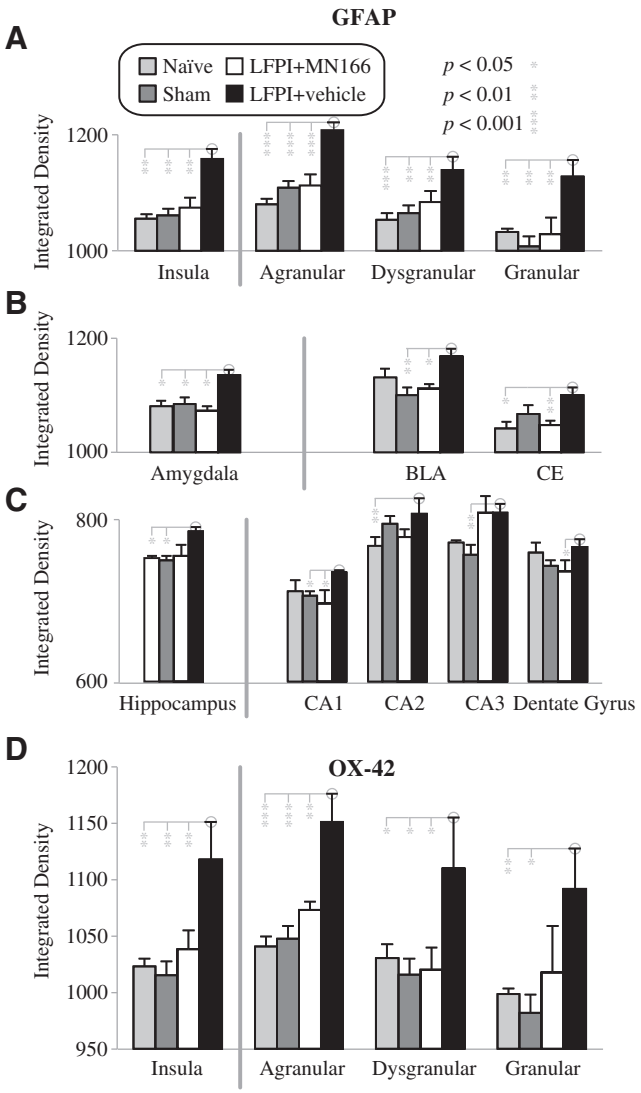

FIG. 4. Regional and sub-regional analyses of microglial and astroglial activation in the hippocampus, amygdale, and insula at 3 months post-injury. (**A-C**) LFPI + vehicle injections induced a significant increase in GFAP labeling in all three regions compared to surgically-naïve, sham-operated, and LFPI + MN166-treated rats. (**D**) In the insula, OX-42 activation was greater in LFPI rats than in surgically-naïve, sham-operated, and LFPI + MN166-treated rats. There were no significant differences found between surgically-naïve, sham-operated, and LFPI + MN166-treated rats in either analysis. Data represent mean ± standard error of the mean integrated densities of immunoreactivity (LFPI, lateral fluid percussion injury; GFAP, glial fibrillary acidic protein; BLA, basolateral amygdala; CE, central amygdala).

first responders to pathogens and neuronal insults in the CNS. They react rapidly, leading to activation of astrocytes and prolonged disruption of neuronal function (Herber et al., 2006; Iravani et al., 2005; Zhang et al., 2009,2010). Several lesion paradigms have also shown a rapid microglial response, followed by delayed astrocyte reaction (Dusart and Schwab, 1994; Frank and Wolburg, 1996; Gehrmann et al., 1991; Liberatore et al., 1999; McCann et al., 1996).

Our results support this well-documented temporal relationship, suggesting that microglial activation precedes astrocytic activation and plays a role in the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; Hanisch, 2002; Herber et al., 2006; Iravani et al., 2005; McCann et al., 1996; Zhang et al., 2010). This time course is consistent with behavioral freezing responses in the present study, appearing rapidly within 2 weeks, but persisting unabated for the 3-month post-injury measurement period. It is also consistent with our immunohistochemistry results, indicating injury-induced astrocytic activation in all three regions of interest, the insula, amygdala, and hippocampus, at 3 months post-injury, but less activation of microglia, which was only significant in the insula. The lower levels of microglia expression are likely due to assessment at 3 months post-injury.

Trauma-related reactive gliosis is well known to result in the release of high levels of proinflammatory cytokines, specifically tumor necrosis factor-α (TNF-α; Fan et al., 1996; Lloyd et al., 2008; Taupin et al., 1993), interleukin-1β (IL-1β; Fan et al., 1995; Fassbender et al., 2000; Lloyd et al., 2008; Taupin et al., 1993; Yan et al., 2002), and interleukin-6 (IL-6; Lloyd et al., 2008; Taupin et al., 1993; Yan et al., 2002), which are central mediators of neuroinflammation following head injury (Fan et al., 1995,1996; Rothwell and Hopkins, 1995; Rothwell and Strijbos, 1995; Simi et al., 2007). Release of these proinflammatory cytokines, particularly IL-1β and TNF-α, pathologically increases neuronal excitability in all brain regions where it has been measured (Beattie et al., 2010; Maroso et al., 2010; Riazi et al., 2008; Rodgers et al., 2009; Schafers and Sorkin, 2008). While neuronal excitability and proinflammatory cytokine levels were not measured in the present study, neuroinflammation has been implicated in neuronal excitability of the amygdala and insular cortex and anxiety-like behavior by others using c-Fos labeling (Abrous et al., 1999; Ikeda et al., 2003; Kung et al., 2010). These same regions have also consistently been reported to be hyperexcitable in human imaging data across a variety of anxiety disorders (Carlson et al., 2011; Rauch et al., 1997; Shin and Liberzon, 2010; Shin et al., 2006; Simmons et al., 2006; Stein et al., 2007).

Attenuation of post-traumatic anxiety with MN166

Meta-analysis of the impact of pharmacological treatments on behavioral, cognitive, and motor outcomes after TBI in rodent models (Wheaton et al., 2011) indicates that of 16 treatment strategies evaluated to date, improved cognition and motor function have been reported, but few treatments have improved behaviors related to psychiatric dysfunction in general, and anxiety in particular. Exceptions to this are recent promising reports of treatments such as magnesium sulfate to limit excitotoxic damage (Fromm et al., 2004; O'Connor, 2003; Vink et al., 2003), and resveratrol to limit excitotoxicity, ischemia, and hypoxia (Sönmez et al., 2007), both increasing open-field exploration (resulting from decreased freezing), and therefore presumably decreasing post-injury anxiety.

Glial-targeted immunosuppression has also been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008), including recent reports that MN166 significantly attenuated brain edema formation, cerebral atrophy, and apoptosis in neuronal cells following ischemic brain injury in rats, increasing neuronal survival rates (Lee et al., 2011). MN166 may reduce neuronal damage in regions

involved in anxiety, mitigating the role of glial activation, neurotoxicity, and hyperexcitability in the subsequent development of anxiety-like behaviors. While not focused on post-traumatic anxiety, MN166 has been found to reduce intracellular calcium accumulation (Yanase et al., 1996), apoptosis, functional damage, and passive avoidance behaviors, following a transient ischemia model in rats (Yoshioka et al., 2002). Increasing evidence supports neuroinflammation, chronic inflammatory responses, proinflammatory cytokines, neuronal hyperexcitability, and secondary injury cascades in the pathophysiology of post-traumatic anxiety. The mechanisms of the effect of MN166 on TBI-induced anxiety-like behavior are not fully known. However, the results of this study provide evidence of a neuroprotective role for MN166 in attenuating and perhaps preventing development of post-traumatic anxiety.

Further exploration of the relationship between TBI, neuroimmune responses, neurocircuitry, and anxiety disorders, is important to better understand the sequelae of TBI, and will aid in the development of effective treatment strategies. The development of anxiety disorders following TBI is a complex and multifaceted problem, and finding treatments that work will require multifaceted approaches. The injury itself initiates many complex biological events, including glial activation, breakdown of the blood–brain barrier, excitotoxicity, and chronic neuroinflammation. While the primary injury often cannot be prevented, it may be possible to reduce the sequelae of secondary injury, leading to better functional and behavioral recovery following TBI. The present results, using peri-injury treatment with MN166 to prevent post-traumatic freezing behavior, not only suggest a role for neuroimmune inflammation in anxiety physiology, but similarly successful results with post-injury treatment could result in clinically-useful new agents to prevent post-traumatic anxiety in humans.

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Author Disclosure Statement

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