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Experimental Traumatic Brain Injury Induces a Pervasive Hyperanxious Phenotype in Rats

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Abstract

Mood disturbances, including depression and anxiety disorders, are common and disabling long-term sequelae of traumatic brain injury (TBI). These psychiatric conditions have generally been considered psychosocial consequences of the trauma, but neurobiological alterations and causes have also been implicated. Using a rat model of TBI (lateral fluid-percussion injury), this longitudinal study seeks to assess anxiety and depression-like behaviors following experimental TBI. Male Wistar rats ($n = 20$) received a severe (~3.5 atmosphere) pressure pulse directed to the right sensorimotor cortex, or sham surgery ($n = 15$). At 1, 3, and 6 months following injury, all rats underwent four assessments of anxiety and depression-like behaviors: exposure to an open field, elevated plus maze test, the forced swim test, and the sucrose preference test. Injured animals displayed increased anxiety-like behaviors throughout the study, as evidenced by reduced time spent ($p = 0.014$) and reduced entries ($p < 0.001$) into the center area of the open field, and reduced proportion of time in the open arms of the plus maze ($p = 0.015$), compared to sham-injured controls. These striking changes were particularly evident 1 and 3 months after injury. No differences were observed in depression-like behaviors in the forced swim test (a measure of behavioral despair) and the sucrose preference test (a measure of anhedonia). This report provides the first evidence of persistent anxiety-like disturbances in an experimental model of TBI. This finding indicates that the common occurrence of these symptoms in human sufferers is likely to have, at least in part, a neurobiological basis. Studies in this model could provide insight into the mechanisms underlying affective disturbance in brain-injured patients.

Key words: anxiety; behavior; depression; rat; traumatic brain injury

Introduction

Following a traumatic brain injury (TBI), patients commonly suffer from a range of long-term neuropsychiatric disturbances, resulting in major impairments in the quality of life experienced by sufferers. These disturbances include cognitive impairments, mood disorders such as depression, anxiety disorders, psychosis, and behavioral problems, such as personality change (Rao and Lyketsos, 2000), and can occur even following a mild insult (Moore et al., 2006). The prevalence of a clinically significant psychiatric disturbance following TBI has recently been estimated to be as high as 50%, compared with 18% for the general population (Fann et al., 2004). Mood disorders are particularly common (van Reekum et al., 2000; Rogers and Read, 2007), with the incidence of depression following TBI reported as 6–77% (Jorge and Starkstein, 2005) and anxiety disorders as 11–70% (Granacher, 2003). As time passes after the injury, there is a decreased probability of suffering from mood disturbance (Ashman et al., 2004), but the prevalence of psychiatric disorders continues to be significantly higher in TBI patients than in control groups even many years after the traumatic injury (Holsinger et al., 2002; Koponen et al., 2002). Despite the extensive literature detailing the high prevalence of affective disorders following TBI, the pathophysiological mechanisms responsible have not been studied in detail.

Animal models are potentially powerful tools to investigate the neurobiological underpinnings of the psychiatric consequences of TBI. As in general biological psychiatry, what are most usefully studied when investigating affective

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disturbance following TBI are endophenotypes (Gould and Gottesman, 2006), rather than comprehensive models of entire disorders such as depression. For example, a cardinal symptom of human depression is despair or hopelessness, an endophenotype modelled by the forced swim test in which a rodent is placed in an inescapable cylinder of water: the animal initially engages in vigorous escape movements, but after some minutes "gives up," becoming increasingly immobile (Porsolt et al., 1977). Likewise, anxiety can be assessed in rodents by taking advantage of the conflict between their natural desire to explore their surroundings and their fear of open spaces. Tests such as the elevated plus maze assess anxiety by measuring parameters such as the time an animal spends in the exposed areas (Carobrez and Bertoglio, 2005).

Despite the high prevalence of psychiatric disorder following human TBI, few studies have addressed the issue using animal models, and those that have been published have reported inconsistent results (Hogg et al., 1998; O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004; Cutler et al., 2006). The purpose of this study was to examine whether affective disturbance occurs following experimental brain injury, and to investigate the temporal development of this disturbance after injury. It was hypothesized that the presence of measurable affective disturbances following experimental brain injury would indicate that these have, at least in part, a neurobiological basis. A variety of measures of anxiety and depressive-like behaviors, well-established as endophenotypes of clinical psychiatric disorders, were assessed following lateral fluid-percussion injury (FPI) in the rat (Thompson et al., 2005). This models moderate to severe closed head injury in humans, producing both focal and diffuse brain damage in survivors, including focal contusion, blood-brain barrier disruption, local and remote axonal injury and progressive neuronal loss, as well as post-traumatic epilepsy in a proportion of animals (Thompson et al., 2005). The delayed development of the post-traumatic epilepsy, with an incidence of ~40–50% at 7–8 months after FPI (Pitkanen et al., 2006), reflects the progressive nature of the neurobiological changes set in train by the acute injury.

Methods

Animals

Male Wistar rats (8–12 weeks old at time of surgery) obtained from our inbred colony at the Royal Melbourne Hospital Biological Research Facility were used in all experiments. Rats were housed individually for the duration of the experiment in this facility under a 12-h light/dark cycle (lights on at 6 a.m.) with *ad libitum* access to food (standard rat chow) and water. All experimental procedures were previously approved by the Pathology, Anatomy & Cell Biology, Microbiology & Immunology, Dental Science & Medicine (RMH) Animal Ethics Committee at the University of Melbourne.

Surgical procedure and lateral fluid-percussion injury

Lateral FPI was applied in line with the well-established procedures for this model (Thompson et al., 2005). The animals were anesthetized by inhalation of isoflurane in equal parts of medical air and oxygen (5% induction, 1.5–2.5% maintenance). A midline scalp incision was made and the underlying periosteum dissected. The scalp and left temporal muscle were reflected, exposing the skull. A 5-mm-diameter craniotomy was performed with a trephine that attached to a drill bit of an electrical drill. The center of the craniotomy was positioned 4 mm lateral and 4 mm posterior to bregma. A modified female Leur-Lock cap was fixed and additionally held in place by cyanoacrylate adhesive and dental acrylic. Subsequently, the injury was induced by a pressure pulse (severe intensity: amplitude of 3.2–3.5 atmospheres) (McIntosh et al., 1989) delivered by a fluid-percussion device (custom-made internally) over 21–23 msec through the Luer-Lock. The fluid pulse from piston plunger (that the pendulum impacts) was conducted via continuous saline fluid into the dura of the rat, ensuring efficient transmission of the pressure pulse. Sham controls received identical surgery without application of the fluid pulse. The lateral position of the injury delivery to the cortex minimized direct involvement of the hippocampus, with this and other structures being secondarily affected by the force transmitted through the brain. During the surgical procedure, animals were connected to a pulse-oximeter by foot clip to enable continuous measurement of heart rate and blood/oxygen levels, indicating depth of anesthesia.

Acute neurological assessment

Acute neurological injury was assessed in all rats on the day prior to and on every day for 3 days after surgery using a composite neuromotor score adapted from McIntosh et al. (1989). Assessments in the neuroscore include ability to traverse a flat wooden beam of 2, 4, and 6 cm width; ability to grip all four limbs onto a horizontal round rod of diameter 1 cm; right and left forelimb flexion observed when the rat is elevated by its tail; presence of a reflex to a loud startle; and the light escape task where the rat must move out of a brightly lit box within 60 sec. Each task is judged on an ordinal score of 0 (pass) or 1 (fail), except the forelimb flexion, which has an ordinal graded severity (maximum deficit score of 2 for each limb).

Behavioral assessments

All rats underwent a series of tests to assess levels of anxiety- and depression-like behavior. The four tests (except the forced swim test, as described below) were performed at 1, 3, and 6 months after injury on separate days in the same order as described. The experimental delay after injury (1 month) was designed to avoid the effects of any transient motor impairment following the injury. For each test session, rats were brought into the behavioral testing suite at Royal Melbourne Hospital at least 30 min prior to assessment (all tests commenced at approximately 2 p.m.), except for the sucrose preference test, which was performed in the housing facility.

1. Open field test. The open field is a 1-m-diameter circular arena enclosed by 20-cm walls with an inner circle (66-cm-diameter) and is widely used as a test of anxiety-like behavior (Prut and Belzung, 2003; Jones et al., 2008). The lighting at the center of the arena was set at ~90 lux, and each rat was placed gently into the center of the field and allowed to explore the arena for 10 min, under continuous videorecording from a camera placed directly above the cen-

ter of the arena. Ethovision Tracking Software (Noldus, Netherlands) was used to quantify the total distance travelled, the number of center entries, and the time spent in the center area.

2. Elevated plus maze. The elevated plus maze is a widely used, ethologically relevant test that assesses anxiety states in rodents (Carobrez and Bertoglio, 2005; Salzberg et al., 2007). Briefly, each rat was placed in the center of a raised plus-shaped maze with two opposite arms enclosed with walls and the other two arms exposed. The lighting in the middle of the maze was set at ~90 lux. The animal was allowed to explore freely for 10 min, during which time its movement was video-tracked from directly above. Quantification of the total distance travelled, the number of entries made, and the time spent in each arm of the maze was assessed using Ethovision Tracking Software.

3. Forced swim test. The forced swim test is commonly used in rodents to measure depression-like behavioral despair (Porsolt et al., 1977; Lahmame et al., 1997). As this procedure is moderately stressful to the animal, it was only performed at the 6-month time point to avoid any potentially confounding effects that this may have on long-term outcomes following injury (Salzberg et al., 2007). The testing apparatus consisted of a clear Perspex cylinder (diameter 30 cm and height 40 cm) filled to a depth of 30 cm with water at 25°C. A training session was performed on day 1, whereby each rat was placed in the cylinder for 15 min. On day 2, in the definitive test session, each rat was placed in the water for 5 min. Both sessions were video-recorded from a horizontal angle, and the test session was later assessed off-line for various behaviors. Time spent in each behavior was summed for the duration of the trial, and the following behaviors were assessed: (i) the time each rat spends in immobility (the primary outcome), defined as making only those movements necessary to keep its head above water; (ii) the time spent climbing, defined as vigorous vertical movement with all four limbs; and (iii) the remaining time of the trial classified as swimming behavior. Only behaviors that persisted for more than 2 sec were scored. Assessments were performed in triplicate by a blinded reviewer on subsequent days and averaged for each rat.

4. Sucrose preference test. The sucrose-preference test is a measure of the "hedonic" state of an animal, or the ability to experience pleasure. Its impairment is a fundamental feature of clinical depression (APA, 2000), and assessment of sucrose preference has been extensively used as a measure of anhedonia in rats (Willner et al., 1987). Animals were presented with two identical drinking bottles, one containing a 1% sucrose solution and the other normal tap water for a 24-h period (start at approximately 10 a.m.). The position of the bottles was randomly assigned to prevent any influence of place preference. The bottles were carefully weighed prior to, and following the 24-h period, and the percentage of the sucrose solution consumed was calculated.

Histological analysis

After the 6-month behavioral exams, animals were given an overdose of Lethabarb *ip* (0.1 mL/100 g) and transcardially perfused with 150 mL of 0.1M phosphate-buffered saline (PBS; pH 7.2) followed by 450 mL of 4% paraformaldehyde (PFA; in 0.1M PBS, pH 7.2) as per our previous study (Kumar et al., 2007). Brains were excised and stored in PFA for 24 h, then immersed in 70% ethanol until undergoing a 24-h paraffin-embedding cycle. Brains were then immediately imbedded in paraffin wax, and stored at room temperature prior to sectioning at 8 μm on a paraffin microtome. Sections were stained in 0.1% thionin (in distilled water; dehydrated and coverslipped). Images were acquired using a wide-field lens and Pentax K10D SLR camera using "Remote Assistant" v3.0 with a Mac Mini Power PC processor; a Leitz 50-mm f4 Photar lens with an aperture setting of 5.6 was used.

Statistical analysis

Analyses for all behavioral variables used analysis of variance (ANOVA) with repeated measures (time after injury), with injury status being the independent variable, with the exception of the Forced Swim test which was only conducted at one time point, and utilized Student's unpaired *t*-tests to compare variables between groups. Bonferroni's analysis was then performed when appropriate, to determine post-hoc significance at individual time points. Data was analyszd using Statistica Software® and, in all cases, statistical significance was set at $p < 0.05$.

Results

Acute effects of fluid-percussion injury

The average intensity of the fluid pulse delivered to animals in the injured group was 3.45 ± 0.06 atm (mean ± SEM). Immediately following this severe impact, all rats experienced a period of apnea lasting approximately 30 sec. Eleven out of 31 (35%) rats did not recover from immediate impact of the injury. Neuromotor scoring deficits were observed in all injured rats ($F_{(1, 35)} = 36.38$, $p < 0.0001$; Fig. 1), which persisted for 3 days after injury. Sham-injured animals showed no deficit in this assessment.

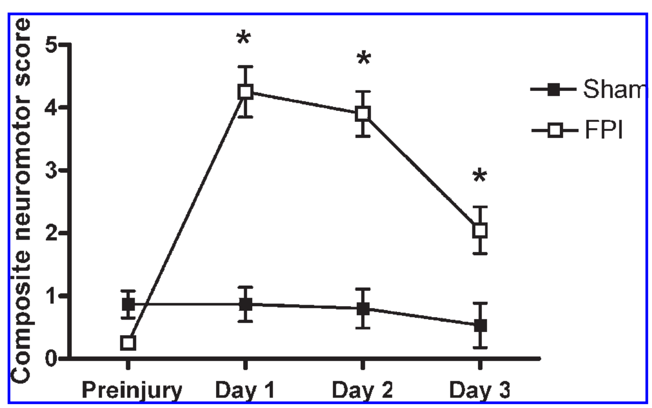

FIG. 1. Fluid-percussion injury (FPI; solid symbols, $n = 20$) causes an acute neurological deficit in rats up to 3 days after impact, as assessed with a composite neuromotor score (*$p < 0.05$). Sham-injured animals (open symbols; $n = 15$) display no deficit. Data represent mean ± SEM.

Fluid-percussion injury induces persistent anxiety-like behavior in the open field test

Overall, the time spent exploring the center of the open field was significantly reduced in animals experiencing a traumatic insult ($F_{(1, 35)} = 6.671$, $p = 0.014$; Fig. 2A), and on post-hoc testing, this reached significance at 1 and 3 months following injury ($p < 0.05$), and neared significance at 6 months ($p = 0.085$). The number of entries made into the inner circle was also significantly reduced in injured animals compared to sham controls ($F_{(1, 35)} = 16.054$, $p < 0.001$; Fig. 2B), and also achieved significance post-hoc testing at 1 and 3 months following injury ($p < 0.05$), and neared significance at 6 months ($p = 0.074$). No differences in total distance travelled were observed between treatment groups ($F_{(1, 35)} = 0.131$, $p = 0.720$; Fig. 2C), indicating the injury did not affect exploratory activity.

Fluid-percussion injury induces persistent anxiety-like behavior on the elevated plus maze

The percentage of time spent in the open arms of the plus maze, an index of anxiety, was significantly reduced in animals following FPI, compared to shams ($F_{(1, 35)} = 6.561$, $p = 0.015$; Fig. 3A), and this reduction reached post-hoc significance at 1 and 3 months following trauma ($p < 0.05$), but had dissipated by 6 months ($p = 0.828$). The percentage of entries into the open arms was also significantly reduced in injured animals ($F_{(1, 35)} = 4.276$, $p = 0.046$; Fig. 3B), and this also was significantly different at 1 and 3 months after injury via post-hoc analysis. Total distance was not affected by injury status in this test ($F_{(1, 35)} = 0.581$, $p = 0.451$; Fig. 3C).

Fluid-percussion injury does not influence behavioral despair in the forced swim test

No differences were observed in the time spent in immobility between injury groups ($t_{(24)} = 0.065$, $p = 0.949$; Fig. 4). Furthermore, other behaviors, including the time spent climbing ($t_{(24)} = 0.917$, $p = 0.368$) and time spent swimming ($t_{(24)} = 0.877$, $p = 0.389$), were not affected by injury condition.

Fluid-percussion injury does not influence anhedonia-like behavior in the sucrose preference test

The total amount of fluid consumed in the 24-h test session was not affected by injury status ($F_{(1, 35)} = 0.201$, $p = 0.656$). Furthermore, no differences between injured animals and sham controls were observed in the preference for sucrose solution at any time point ($F_{(1, 35)} = 0.006$, $p = 0.940$; Fig. 5).

Histological assessment

FPI induced marked cortical atrophy; distortion and atrophy of the hippocampus and thalamus; and ventricular swelling in all rats (Fig. 6). This damage was evident beyond the extent of the initial injury site. Contralateral brain structures appeared to be morphologically preserved, although lateral ventricular enlargement was observed in some injured rats contralateral to injury. The surgery performed on sham-injured rats resulted in mild cortical atrophy and slight ventricular enlargement when examined 6 months post-surgery. This was evident in all sham-injured rats but was limited to the region of the craniotomy, and was not as severe as rats sustaining the fluid-pressure pulse.

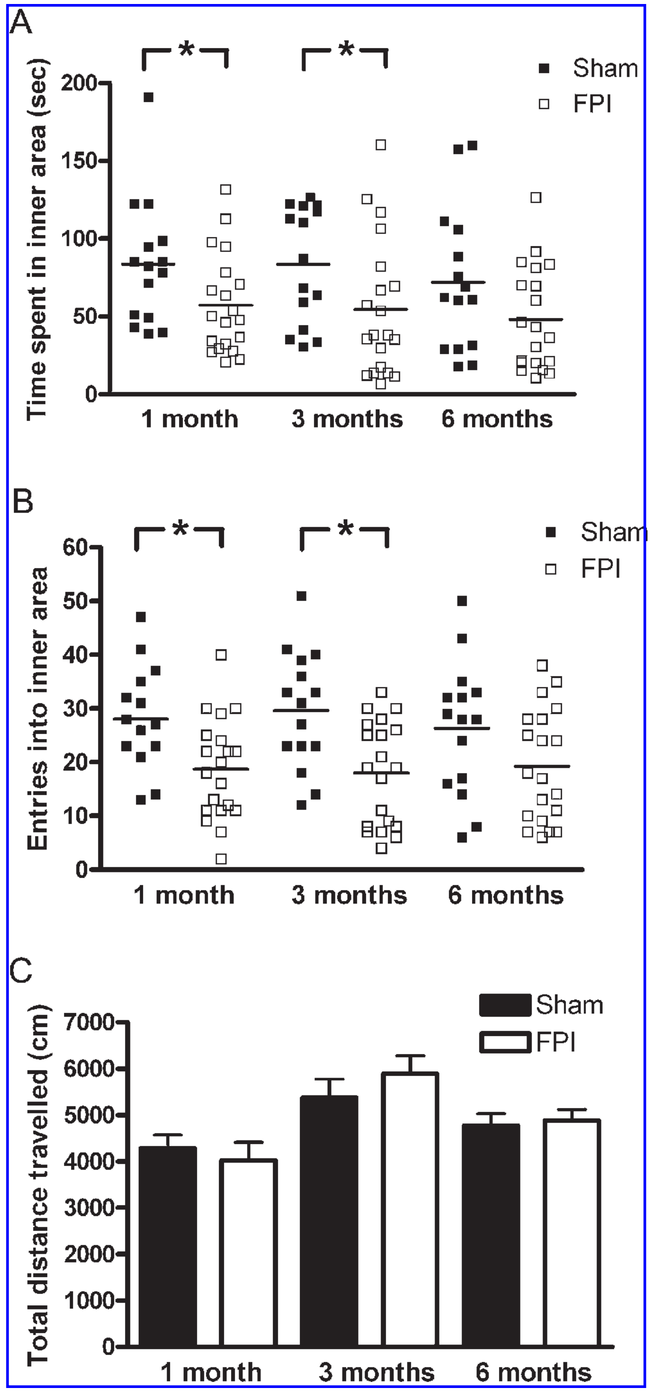

FIG. 2. Fluid-percussion injury (FPI; solid symbols, $n = 20$) increases anxiety-like behavior in the open field test, as evidenced by significantly reduced time in the inner area (**A**) and significantly reduced entries into the inner area (**B**; $*p < 0.05$), compared with sham-injured animals (open symbols; $n = 15$). (**C**) No differences are observed in total distance travelled. A and B depict scatterplots of individual values, and horizontal lines represent the means, whereas data in C represent mean + SEM.

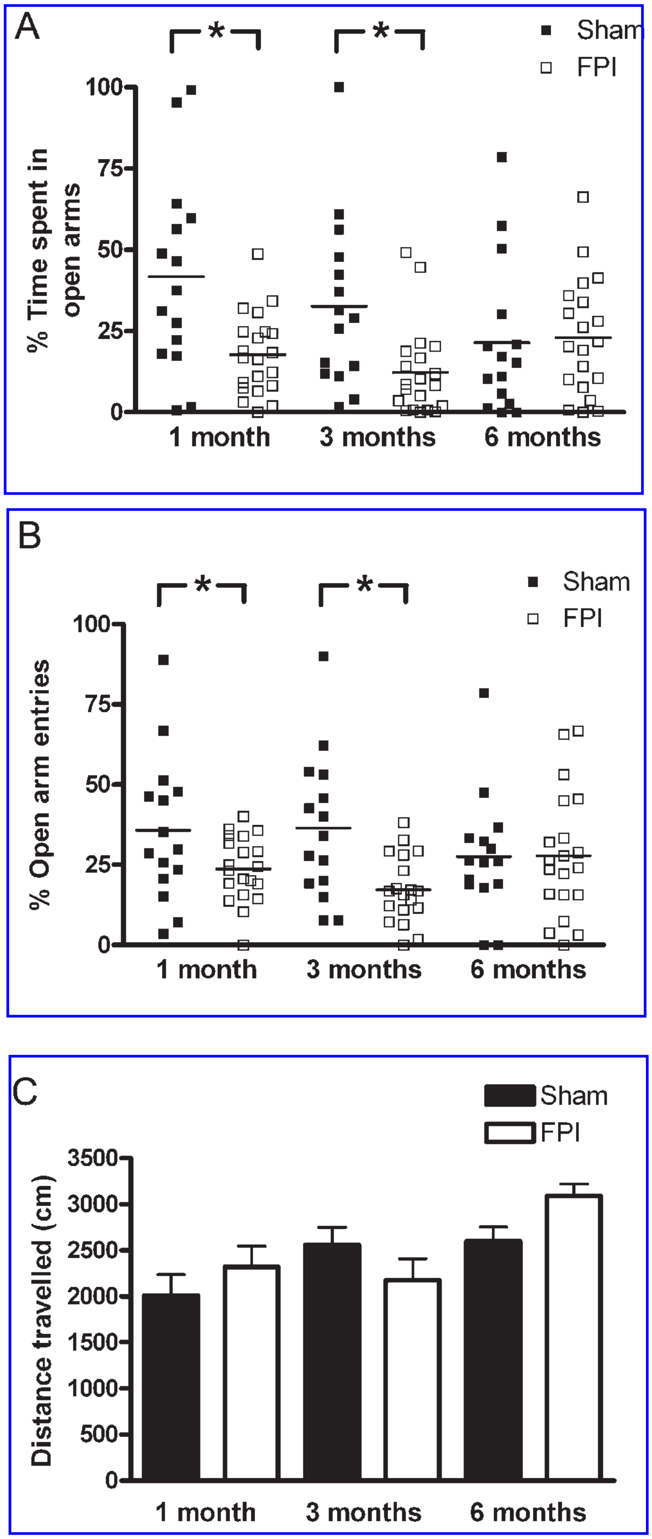

FIG. 3. Fluid-percussion injury (FPI; solid bars, $n = 20$) increases anxiety-like behavior in the elevated plus maze test, as evidenced by significantly reduced percentage time in the open arms (**A**) and significantly reduced percentage entries into the open arms (**B**; $*p < 0.05$), compared with sham-injured animals (open bars; $n = 15$). (**C**) No differences are observed in total distance travelled. A and B depict scatterplots of individual values, and horizontal lines represent the means, whereas data in C represent mean + SEM.

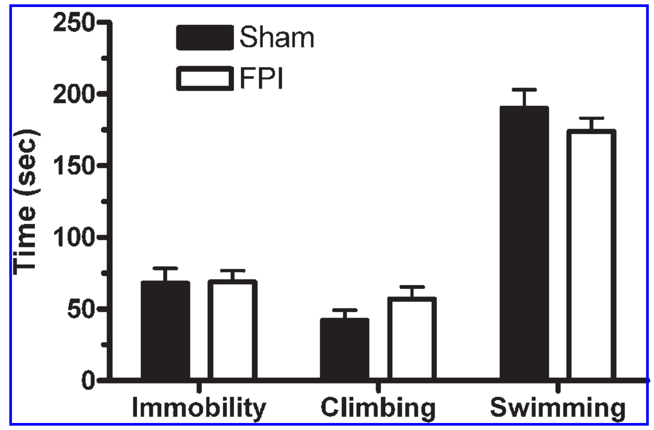

FIG. 4. Fluid-percussion injury (FPI) does not affect depression-like behavior in the forced swim test. No significant differences in time spent immobile, time spent climbing, or time spent swimming are observed after injury ($p > 0.05$) between injured (solid bars; $n = 20$) and sham-injured (open bars; $n = 6$) rats 6 months following injury. Data represent mean + SEM

Discussion

Here we report the presence of persistent anxiety-like behaviors following an experimental model of TBI. This observation has important clinical implications, suggesting that the common occurrence of anxiety disorders in humans following trauma may, at least in part, have a neurobiological basis. Additionally, it defines the lateral FPI model in the rat as one that can be utilized to investigate the pathophysiology of anxiety disorders following TBI. There is now abundant literature detailing the increased prevalence of affective disorders following TBI in humans (van Reekum et al., 2000; Rogers and Read, 2007), disturbances that contribute greatly to disability and to impaired quality of life. While 18% of the general population are estimated to experience psychiatric disorders, the prevalence of mood disturbance following TBI has been reported as high as 77% for depression (Jorge and Starkstein, 2005) and 70% for anxiety disorders (Granacher, 2003), which are significant and clear elevations from the norm.

Despite the high prevalence and importance of psychiatric disturbances in human TBI sufferers, this issue has largely been ignored in fundamental trauma research using rodent models, which has tended to focus on cognitive, sensory, and/or motor deficits following injury (Thompson et al., 2005). Indeed, to our knowledge, this is the first published study reporting persisting changes in anxiety-like behavior. Depression is also a common occurrence following brain injury in humans; thus, it is of interest that two standard measures of depression-like behavior did not show changes in our study, but have been reported up to 90 days after injury elsewhere (Milman et al., 2005; Shapira et al., 2007).

Differences in results between our study and the few others that have addressed the issue may be due to differences in species studied, method and severity of experimental TBI, timing of the affective measures following injury, and other methodological differences. Two studies have reported no differences in anxiety-like behaviors after traumatic injury

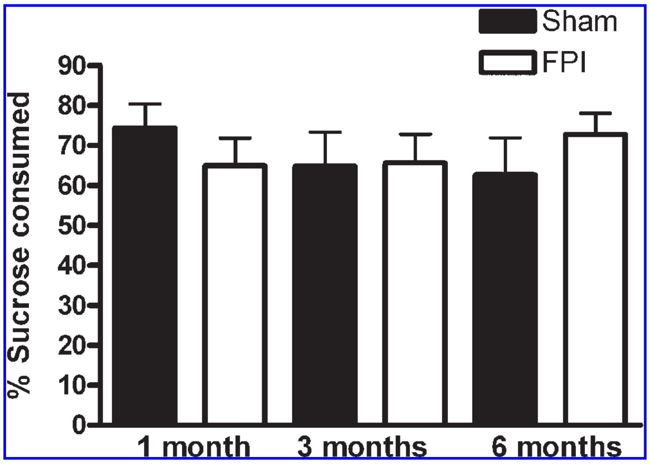

FIG. 5. Fluid-percussion injury (FPI) does not affect depression-like behavior in the sucrose-preference test. No differences in sucrose preference are observed at any time point after injury ($p > 0.05$) between injured (solid bars; $n = 20$) and sham-injured (open bars; $n = 15$) rats. Data represent mean + SEM.

(Hogg et al., 1998; Cutler et al., 2006), but these assessments were made only up to 1 week following injury. The acute motor deficits that not uncommonly persist this long following FPI may have confounded the ability of the investigators to detect an increase in anxiety-like behaviors at this early time point. Furthermore if neuronal plastic changes post-injury are required to cause altered anxiety states (Cannistraro and Rauch, 2003), then it would be expected that, following injury, structural and functional reorganization would take longer than 1 week to manifest. A small body of published work details decreased exploratory activity in the open field test in rats after TBI using the weight-drop model (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004), a behavior interpreted by those authors as increased "depression-like behavior," although this test is more commonly and correctly described as a measure of anxiety-like behavior, as it is here (Prut and Belzung, 2003). Another series of papers detailed *increased* locomotor activity in the open field test in gerbils, suggesting that behavioral effects of brain injury may be species-dependent (Li et al., 2006a,b). While these reports describe effects of TBI on general motor activity, they make no attempt to assess the influence of the aver-

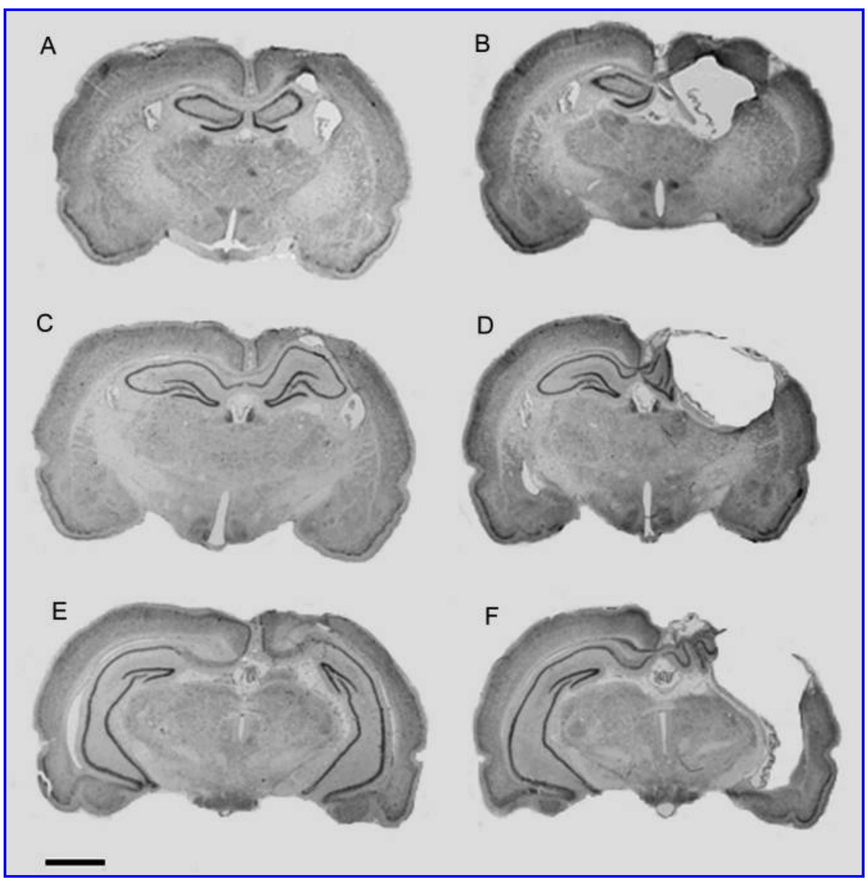

FIG. 6. Coronal thionin-stained histological images of a representative sham-injured rat brain (**A**,**C**,**E**) and a representative traumatically injured rat brain (**B**,**D**,**F**) at 6 months post-injury. Significant ipsilateral cortical and hippocampal damage is apparent with extensive cavitation and pannecrosis following fluid-percussion injury (FPI). Some cortical atrophy is also observed following sham injury in this model. Scale bar = 2 mm (and applies to all images).

sive nature of the open field task (i.e., which specific areas of the maze the rodent explores). It is this aspect that makes these tests assessments of anxiety, rather than of general activity.

The causation and pathogenesis of mood and anxiety disorders following TBI remain to be elucidated. Inflammation, repair (including neural regeneration and gliosis), and neuroplastic structural and functional reorganization of neural circuits could all be relevant, as could alterations in neuroendocrine function and interruption to neurotransmitter pathways implicated in affective disorder. Inflammatory cytokines have been implicated in post-stroke depression (Spalletta et al., 2006) and may well be relevant to post-traumatic affective disturbance. The fact that brain trauma can lead to post-traumatic epilepsy in at least 10% of cases, usually beginning months or years after the injury (Hauser et al., 1991; Englander et al., 2003), illustrates the presence of neuroplastic changes, particularly in hippocampus and amygdala (Pitkanen et al., 2006), which are structures also implicated in affective disorder. Histological analysis of post-mortem samples in this study details severe damage, tissue atrophy, and cell loss in the hippocampus, thalamus, and somatosensory cortex, as well as ventriculomegaly, as previously described (Smith et al., 1997), suggesting these areas may be involved in the behavioral pathology observed. It is interesting to note that some cortical atrophy was also observed at 6 months in the sham-injured animals. While this has not been clearly described in the literature, there is evidence that suggests the sham surgery (i.e., craniotomy, anaesthesia) does indeed produce deleterious effects: some have reported seizures in sham-injured animals (Carbonell and Grady, 1999; Blaha et al., 2000), and others have detailed reactive gliosis at the site of the craniotomy (Carbonell and Grady, 1999).

Long-term changes in neurotransmitter systems may also be involved in the development of mood disorders following trauma, since many, predominantly serotonin and norepinephrine, but also others such as glutamate, dopamine, γ-aminobutyric acid (GABA), and acetylcholine (Janowsky et al., 1994; Singh et al., 2004; Belmaker and Agam, 2008) are strongly implicated in psychiatric disorders, and these neurochemical systems can be greatly disturbed by TBI (Dixon et al., 1997; Ciallella et al., 1998; Wilson and Hamm, 2002). Further research is needed to ascertain whether some, or all of these immunological, structural, and functional alterations are involved in the pathogenesis of affective disturbance following TBI, but the model and experimental paradigm reported here would be a suitable tool for such research.

To conclude, here we demonstrate long-lasting anxiety-like mood disturbances in a rat model of TBI. These disturbances begin as early as one month following injury but appear to diminish by 6 months. Interestingly, we did not find changes in depressive-like behavior. Despite this dichotomy, which deserves further research, our findings support a neurobiological basis for the anxiety-like symptoms commonly experienced by humans following TBI.

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Author Disclosure Statement

No competing financial interests exist.

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