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Acute Cognitive Impairment After Lateral Fluid Percussion Brain Injury Recovers by One Month: Evaluation by Conditioned Fear Response

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Abstract

Conditioned fear associates a contextual environment and cue stimulus to a foot shock in a single training trial, where fear expressed to the trained context or cue indicates cognitive performance. Lesion, aspiration or inactivation of the hippocampus and amygdala impair conditioned fear to the trained context and cue, respectively. Moreover, only bilateral experimental manipulations, in contrast to unilateral, abolish cognitive performance.

In a model of unilateral brain injury, we sought to test whether a single lateral fluid percussion brain injury impairs cognitive performance in conditioned fear. Brain-injured mice were evaluated for anterograde cognitive deficits, with the hypothesis that acute injury-induced impairments improve over time. Male C57BL/6J mice were brain-injured, trained at five or 27 days post-injury, and tested 48 hours later for recall of the association between the conditioned stimuli (trained context or cue) and the unconditioned stimulus (foot shock) by quantifying fear-associated freezing behavior. A significant anterograde hippocampal-dependent cognitive deficit was observed at seven days in brain-injured compared to sham. Cued fear conditioning could not detect amygdala-dependent cognitive deficits after injury and stereological estimation of amygdala neuron number corroborated this finding. The absence of injury-related freezing in a novel context substantiated injury-induced hippocampal-dependent cognitive dysfunction, rather than generalized fear. Variations in the training and testing paradigms demonstrated a cognitive deficit in consolidation, rather than acquisition or recall. By one month post-injury, cognitive function recovered in brain-injured mice. Hence, the acute injury-induced cognitive impairment may persist while transient pathophysiological sequelae are underway, and improve as global dysfunction subsides.

Keywords

Head Injury; TBI; Amygdala; Hippocampus; Mouse; stereology; disector; fractionator

Introduction

Traumatic brain injury (TBI) is the leading cause of morbidity and mortality in the United States [64,78]. Brain-injury survivors often suffer cognitive deficits, including impaired learning and memory [10,61], arising from specific damage to the temporal lobe and

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hippocampus [3,29]. Additionally, components of post-concussion syndrome, including impaired affective and emotional function, may result from pathophysiology in the extended limbic system, including the amygdala [61]. Lateral fluid percussion brain injury (FPI) in the rodent reproduces many of the pathological features of human TBI, including neuronal loss, gliosis, metabolic, and ionic perturbations [81], which contribute to an enduring cognitive impairment [67]. However, deficits in amygdala function, giving rise to impaired affect, remain understudied in experimental TBI.

The amygdala and the hippocampus synergistically function to form long-term memories of emotional events [51,73]. The association of the conditioned and unconditioned stimuli during aversive Pavlovian conditioning converges on the limbic system [9,24]. In conditioned fear, subjects come to express defensive responses to a neutral conditioned stimulus (CS) that is paired with an aversive unconditioned stimulus (US). Conditioned fear is acquired rapidly, retained over long periods of time, and is readily studied in both rodents and humans. In patients, unilateral temporal lobectomy produces attenuated conditioned fear responses [48], corroborated by rodents studies and extended to demonstrate that bilateral amygdala lesions abolish conditioning [11,47]. In rodents, a foot shock serves as the unconditioned stimulus to elicit a defensive freezing conditioned response. The experimental context or a tone serves as the conditioned stimuli to elicit a fear-associated defensive freezing conditioned response.

Damage to the limbic system, including the amygdala and hippocampus, disrupts the associative processes and expression of Pavlovian conditioned fear [16,84], but not the performance of the fear responses per se [42,55]. Specifically, lesions of the hippocampus selectively impair contextual conditioned fear [46,66], whereas lesions of the amygdala impair both cued and contextual conditioned fear [66]. Hippocampal damage after lateral FPI includes cell loss, glial activation, electrophysiological dysfunction, and a host of gene expression changes [81]. In this way, anterograde deficits in the acquisition or expression of contextual conditioned fear can be demonstrated within one-week after lateralized brain injury in the rat [2,41]. In addition, amygdala damage after experimental brain injury includes dystrophic neurons and immediate early gene expression [2,19]. Although clinical studies describe injury-induced emotional outbursts reminiscent of amygdala damage [61], cognitive performance in cued conditioned fear has yet to be reported after experimental TBI.

Stereological reports have demonstrated a significant non-progressive loss of neurons throughout the hippocampus ipsilateral to unilateral FPI [12,28,87,88], and contralateral neuronal damage can be detected by fluorojade-positive staining [36,75]. Systematic neuropathology within the amygdala has yet to be reported, however qualitative histology demonstrates no overt pathology [38]. As a model of both focal and diffuse experimental brain injury, the current set of experiments sought to test whether a single lateral FPI can disrupt performance in conditioned fear. Bilateral lesions permanently disrupt the cellular basis for conditioned fear performance [57], whereas unilateral ablation, chemical lesion or pharmacological inactivation of the hippocampus has been insufficient to eliminate (only attenuate) cognitive performance [11,20,22,25,47,69]. Therefore, cognitive function after lateral FPI may be impaired while the injured brain remains stunned [82], but recovers as bilateral damage subsides.

In the present communication, cognitive function in the traumatically injured brain was evaluated in terms of hippocampal-dependent visual-spatial memory (context) and amygdala-dependent classically-conditioned fear (cue). To evaluate the nature of cognitive function remaining after experimental TBI, conditioned fear was assessed at time points remote from the injury, after pathological cascades subside. By varying the temporal spacing of the training, the brain injury and the testing, the effects of TBI on acquisition, consolidation and recall of a conditioned fear cognitive task could be evaluated. Since, brain-injured mice may be incapable

of recalling an association conditioned immediately prior to brain injury due to retrograde amnesia [88], the present design explores anterograde cognitive function in the injured brain. Furthermore, quantitative stereological analysis of the basolateral amygdala complex and central nucleus were undertaken, since they serve the anatomical basis for amygdala-dependent conditioned fear [59,76].

Materials & Methods

Generation of Animals

Adult male C57BL/6J mice (5-7 wks, 20-25g; Jackson Laboratory, Bar Harbor, ME) were used in all experiments. All experimental procedures and protocols for animal studies were approved by the University of Pennsylvania Institutes for Animal Care and Use Committees (National Research Council, National Academy Press, Washington, DC, 1996). An additional thirteen C57BL/6J naïve mice (no surgery or brain injury) were evaluated in the behavioral paradigm.

Fluid Percussion Injury Surgery: Day 1—Fluid percussion brain injury in the mouse was conducted as previously published [18,88]. Each animal was anesthetized using sodium pentobarbital (65 mg/kg, i.p.). The animal was placed in a mouse stereotaxic frame (Stoelting, Wood Dale, IL). The scalp was reflected with a single incision and the fascia scraped from the skull. All of the following procedures were conducted under 0.7-3.5X magnification. An ultra-thin teflon disc, with the outer diameter equal to the inner diameter of a trephine, was glued with Vetbond (3M, St. Paul, MN) onto the skull between Lambda and Bregma, and between the sagittal suture and the lateral ridge over the right hemisphere. Using a trephine (3 mm outer diameter), the craniectomy was performed, keeping the dura intact. A rigid Luer-loc needle hub (3 mm inside diameter) was secured to the skull over the opening with cyanoacrylate adhesive and dental acrylic. The skull sutures were sealed with the cyanoacrylate during this process to ensure that the fluid bolus from the injury remained within cranial cavity. The hub was capped until Day 2. The animal was sutured, placed on a heating pad, and returned to the home cage once ambulatory.

Fluid Percussion Injury: Day 2—Each animal was placed under isoflurane anesthesia (2% oxygen in 500 ml/minute) via nose cone and respiration was visually monitored. Once the animal reached a surgical plane of anesthesia (one respiration per two seconds), the nose cone was removed, the cap over the hub was removed, and dural integrity was confirmed visually. The hub was filled with isotonic sterile saline and a 32 cm piece of high-pressure tubing from the fluid percussion injury (FPI) device (Custom Design & Fabrication, Virginia Commonwealth University, Richmond, VA) was attached to the Luer-loc fitting of the hub. The animal was placed onto a heating pad on its left side and, once a normal breathing pattern resumed, before sensitivity to stimulation, the injury was induced, imposing a 20 ms pulse of saline onto the dura. The pressure transduced onto the dura was monitored with an oscilloscope, with injury severity ranging between 2.1-2.2 atmospheres. Immediately after injury, the hub was removed from the skull and the animal was placed in a supine position. Sham animals received all of the above, with the exception of the fluid pulse, and were generated in cohorts along with brain-injured mice. The time elapsed until the animal spontaneously righted was recorded as an acute neurological assessment, and defined as the righting reflex time. Animals in the sham group had a mean righting time of 8.4 ± 3.0 seconds ($n = 47$; range 3-17 sec). Injured animals had a mean righting time of 310.8 ± 161.7 seconds ($n = 54$; range 52-720 sec). The animal was then anesthetized under isoflurane to suture the scalp. Animals were returned to a heating pad until ambulatory and then returned to the home cage. Seven brain-injured animals (11.5%) died as a result of the respiratory depression/apnea after injury and were not included in the study.

Hippocampal Aspiration Lesions—Mice were anesthetized (sodium pentobarbital, 60 mg/kg, i.p.) and placed in a stereotactic frame. A 3 mm long, 0.5 mm wide craniotomy was made over the left parietal bone using a dental drill, exposing the neocortex above the hippocampus. Visually guided aspiration lesions were made with an 18G-blunted needle attached to a laboratory vacuum pump, as previously described [40]. During hippocampal aspiration (n = 5), the overlying cortex and white matter are removed to expose the surface of the hippocampus. After surgery, the incision was sutured and mice were placed on a heating pad until ambulatory. Mice were returned to the home cage and allowed 28 days for recovery before the start of conditioned fear training.

Contextual Fear Response

To determine the extent of cognitive function anterograde to the brain injury, brain-injured and sham-injured mice were trained and tested after lateral fluid-percussion brain injury, according to the conditioned fear protocols outlined in Figure 1. All animals received two days of handling (two min, twice a day) prior to conditioning. The training and testing for the conditioned fear experiments were conducted using a computer-controlled HabiTest system (Coulbourn Instruments, Allentown, PA). To minimize variation in behavior, conditioning and testing were conducted at the same time of day, level of room light, and background noise.

During conditioning (acquisition), animals were trained individually in a conditioning cage (17.8 × 17.8 × 30.5 cm, metal and Plexiglas walls, shockable grid floor) within a darkened, sound-attenuated chamber (trained context). The cage was cleaned with 70% ethanol before each experiment and before each animal entered. In the modular conditioning cage, a white-noise generator provided background noise (20 Hz – 20 KHz) throughout the experiment. For weak association conditioning, each mouse was placed in the cage for 90 seconds, after which a 2 KHz tone (cue) was activated for 20 seconds. The tone co-terminated with a foot shock (0.5 mA, 2 sec) delivered through the grid floor. For strong association conditioning, each mouse was placed in the cage for 120 seconds, after which a 3.5 KHz tone and incandescent light (cues) were activated for 30 seconds. The cues co-terminated with a foot shock (1.5 mA, 2 sec) delivered through the grid floor. The cues and foot shock were repeated 30 seconds later. All mice remained in the chamber for an additional 30 seconds after the last foot shock, before being returned to its home cage. Under these training conditions, mice were provided with both context and cue stimuli that are conditioned to the foot shock.

Two days later, recall of hippocampal-dependent contextual conditioning was tested by returning a trained mouse to the trained context without cues for five minutes and scoring fear-associated freezing behavior (conditioned response = freezing). Freezing is defined as a total lack of movement, aside from respiration [46,66], and recorded as seconds of inactivity sampled at 10 Hz using a computer-controlled infrared thermal activity monitor. Recall of amygdala-dependent cued conditioning is tested by placing a trained mouse in a novel context (round, white, plastic container with corn-cob bedding inserted into the conditioning chamber) for two minutes, then activating the cue(s) for the remaining three minutes of the five minute test period. Novel context and cued conditioned fear is performed following context conditioned fear, since the cue association is more robust and more resilient to extinction [6, 15]. The two-minute novel context provides a control for possible confounding effects of the training context, ensuring that the observed freezing was specific to the conditioned context and cues as opposed to other environmental stimuli.

Data are presented as percent freezing, calculated from the seconds of inactivity in each minute of the five minute context test, two minute novel context test and the three minute cue test. In addition, an activity suppression ratio was calculated for 90 seconds of each test to normalize the fear-associated freezing response to each animal's baseline (pre-shock) activity level (see Fig. 1 for baseline and test periods used in quantification). To minimize contamination of the

data by the mouse's own baseline activity, the activity suppression ratio was defined as (activity during test) / (activity during test + activity during baseline) [7]. The activity suppression ratio is based on a reduction in baseline activity to indicate a fear response, rather than freezing, where an activity suppression ratio of 0.5 indicates no fear and values 0.0 to 0.5 indicate high levels of fear. Activity suppression ratio values greater than 0.5 can indicate conditioned safety [7].

Contextual freezing and activity suppression demonstrate cognitive impairments after hippocampal lesions in a conditioned fear test using a computer-aided system [56]. In contrast, the present computer-aided behavioral system does not require video frame analysis algorithms to detect mouse movement or inactivity. The present system meets the criteria established by Anagnostaras et al. for computerized measurement of freezing, and is capable of extracting the activity suppression as an index of fear [7]. In the initial characterization of the equipment, the observer and computer scoring of freezing resulted in a near linear correlation (data not shown). Further evaluation could not be completed, since observer scoring required the sound attenuated chambers to be open, in which case light and sound cues would cross contaminate experiments.

Design-Based Stereology in the Amygdala

One-week or one-month after lateral fluid percussion, animals were euthanatized for histological evaluation. Design-based stereological estimates of neuronal number in the basolateral complex (input), central nucleus (output), and subiculum (hippocampal output) were compared bilaterally in brain-injured and uninjured mice. Stereological estimates of ipsilateral and contralateral neuronal number in hippocampal subregions (dentate gyrus, hilus, area CA3, and area CA1) are concurrently reported from our laboratory [81,88]. Briefly, counting 150-200 neurons in a defined fraction of each tissue section, through a known depth of the tissue section, in a known fraction of tissue sections allows for a mathematical estimate of the total population of neurons, as described below. Neuronal number estimates are independent of brain region or neuronal volume, and any tissue shrinkage or expansion resulting from processing [86].

A random subset of mice ($n = 4$ sham; $n = 7$ injured) were euthanatized by an overdose of sodium pentobarbital (200 mg/kg) and transcardially perfused with heparinized saline followed by fresh 10% neutral-buffered formalin and post-fixed overnight at 4°C. Subsequently, the brains were removed, blocked, subjected to standard paraffin processing (56-58°C melting point) and embedded. Using a Leica RM-2165 rotary microtome (Nussloch, Germany), the brains were sectioned exhaustively at 50 μ m in the horizontal plane, and wet mounted on gelatin-subbed slides. Sections were deparaffinized, rehydrated, stained with 0.05% cresyl-violet, destained in a graded series of ethanol, cleared in xylene, and coverslipped [17].

The basolateral amygdaloid complex, central nucleus, and subiculum were identified based on morphology and clearly identifiable landmarks adapted from horizontal sections of the rat brain [65] and descriptions for the rat amygdala [83]. The basolateral amygdala encompasses the lateral, basolateral and basomedial nuclei of the amygdala. Every second section containing the amygdala, or every fourth containing the subiculum, was selected to systematically sample the nuclei (the section-sampling fraction, *ssf*; amygdala = 0.5, subiculum = 0.25), yielding 9-11 sections per brain. The first of these sections was randomly selected. In the sampled sections, an optical disector counting frame was employed for counting neuronal nuclei at predetermined regular *x,y* intervals [31]. Inclusion and exclusion counting criteria were followed by a blinded observer who recorded counts only when a single neuronal nucleus was brought into focus within the disector frame [79]. Because the area (*a*) of the counting frame was known relative to the regular stage-stepping intervals over the section, one can calculate the area sampling fraction (*asf*) = $a(\text{frame})/a(\text{x,y step})$. The *asf* for the subiculum is 0.0140; the basolateral

complex is 0.0071; and the central nucleus is 0.0218. The height (h ; 35 μm) of the optical disector was known relative to the thickness (t ; 45 μm) of the section. With these parameters, the estimated number of neurons (N) followed from the formula $N = \Sigma Q^- \cdot t/h \cdot 1/\text{asf} \cdot 1/\text{ssf}$, where ΣQ^- was the number of neurons counted.

To analyze the sampling scheme reliability, for every animal, coefficient of error (CE) was calculated using Matheron's quadratic approximation [33] and by considering the "nugget effect" [85] to reflect the variance introduced by the sampling of tissue sections. All sampling was conducted using a microscope (Nikon, Tokyo, Japan), with a 63X, 1.4 numerical aperture oil immersion objective (Leitz, Wetzlar, Germany). A mounted video camera (Toshiba, Tokyo, Japan), and microcator (Heidenhain, Deerfield, IL) with 0.2 μm sensitivity were used in conjunction with Olympus CAST software (Olympus Danmark, Copenhagen, Denmark).

Reagents and Statistical Tests

Reagents were purchased from Sigma (St Louis, MO). Differences in fear-associated freezing between brain-injured and uninjured groups were analyzed by repeated measures ANOVA across the individual minutes, with Neumann-Keuls post-hoc test to identify significant differences at individual minutes. All fear suppression ratio data were analyzed by two-tailed one-sample T-test to determine whether group means were significantly different from 0.5, indicating fear associated with the conditioning paradigm. Differences in the fear suppression ratio were analyzed by two way ANOVA (group $\times$ test condition), with Neumann-Keuls post-hoc tests. Differences in neuronal number were analyzed by two-way ANOVA (hemisphere $\times$ group), separately for each brain region, with Neumann-Keuls post-hoc test to identify specific differences. Significance was ascribed where $p < 0.05$.

Results

Conditioned Fear in Naïve Mice – Experiment 1

In order to establish the conditioning protocols for use in brain-injured animals, naïve mice were subjected to the weak association ($n = 10$) or strong association ($n = 3$) conditioning paradigms. To detect a context-dependent cognitive deficit, the weak association training minimized salient cues (the number of training sessions and shock exposures) in contrast to the strong association training. After two days of handling, naïve mice exposed to the conditioning chamber, before the onset of the conditioned or unconditioned stimuli, demonstrate baseline freezing of $10.1\% \pm 7.7$ (weak association) and $22.9\% \pm 14.5$ (strong association). The inherent variability (coefficient of variation 63%-76%) in the baseline freezing percent supports the use of the baseline-corrected activity suppression ratio, rather than freezing percent. Two days after conditioning, these mice exhibit 3-8 fold increases in freezing to the trained context over baseline up to $84.0\% \pm 13.1$ in the weak and $68.7\% \pm 4.12$ in the strong association conditioning (Fig. 2B), which represent a hippocampal-dependent cognitive task. However, the conditioning does remain specific to the conditioned stimulus, as the freezing exhibited in the first two minutes of the novel context reflects the baseline behavior (weak: $8.0\% \pm 6.8$; strong: $12.8\% \pm 14.7$)(Fig. 2C). Furthermore, in the presence of the conditioned stimulus cues (tone or tone and light for minutes 3 - 5) freezing behavior is significantly elevated over baseline (weak: $59.2\% \pm 27.1$; strong: $71.8\% \pm 28.7$)(Fig. 2C).

To eliminate the inherent variability between mice that is reflected in the baseline freezing data [60], an activity suppression ratio was calculated for each mouse. This ratio provides an indication of suppressed activity in response to the trained context, trained cues and novel context, where a value of 0.5 indicates no fear and as values decrease the degree of fear increases. As expected, activity suppression is not significantly different from 0.5 in the novel context, indicating no generalized fear to the testing situation. The activity suppression ratios

in response to the trained context and cues are significantly lower than 0.5 in the strong conditioning paradigm, but only significant for the context in the weak conditioning paradigm (Fig. 2D). In fact, the differential reductions of fear to the context and cue in mice conditioned with the weak versus strong association paradigms highlight the differences between the conditioning paradigms, namely the saliency of the cue in obscuring the context conditioning. At the expense of cue conditioning, brain-injured mice were subjected primarily to weak association conditioning to minimize interference of strong light and tone cues on the context training.

After establishing the conditioning and testing procedures for naïve mice, nQuery Advisor 5.0 (Statistical Solutions, Saugus, MA) was used to determine the appropriate sample sizes per group. Based on the standard deviations in the activity suppression ratio derived from naïve mice in the weak association group, a sample size of 10 in each group will have 95% power ($\alpha = 0.05$) to detect a change of 0.15. With a mean of 0.145, both increases and decreases in fear can be detected.

Injury-Induced Anterograde Cognitive Deficit Recovers – Experiment 2

To evaluate the time course of anterograde cognitive function in brain-injured mice, paired cohorts of brain-injured and sham-injured animals were trained and tested in the conditioned fear paradigm at one week or one month after brain injury.

By two days after brain or sham injury, all mice were ambulatory, feeding and gaining weight. Brain-injured ($n = 13$) and sham ($n = 12$) mice were subjected to weak association conditioning on the fifth day after brain injury or sham. Freezing behavior during the conditioning was not significantly different between the groups (sham, $15.6\% \pm 9.7$; FPI, $25.3\% \pm 20.2$). Two days later, mice were tested in the trained context and novel context for classical conditioning to the context and cues. Two days after conditioning, brain-injured and sham-injured groups of mice demonstrate fear associated with the conditioned context (activity suppression ratios significantly different from 0.5). The extent of fear expressed to the trained context at one-week post-injury in brain-injured mice is significantly less than sham (Fig. 3A). Using the weak association paradigm, neither group expressed fear associated with the cue stimuli. However, conditioning remained specific to the trained associations, as fear did not generalize to the novel context. In comparison to naïve mice trained under the weak association paradigm, the activity suppression ratios in sham mice are higher, suggesting some degree of behavioral habituation or helplessness associated with the surgery, sham injury and handling.

By one month, the brain-injured mice ($n = 12$) are indistinguishable from sham mice ($n = 10$) when measured by anterograde cognitive performance. Using the same weak association conditioning paradigm, mice were conditioned 26-27 days after injury and tested two days later. Freezing behavior during the conditioning was not significantly different between the groups (sham, $21.0\% \pm 17.5$; FPI, $22.5\% \pm 21.9$). The activity suppression ratios for the context, cue and novel context are not significantly different between sham and brain-injured mice (Fig. 3B). The initial cognitive deficit observed at one week after brain injury had resolved by one month. In comparison to naïve mice trained under the weak association paradigm, the activity suppression ratios in sham and brain-injured mice are comparable, suggesting recovery in both groups of animals.

Nature of Cognitive Deficit at One Week Post-Injury – Experiment 3 & 4

To explore the nature of the post-injury anterograde cognitive deficit evident one week after brain injury, separate groups of mice were evaluated for impairments in acquisition of the weak association conditioning task (Experiment 3) or the retention of strong association conditioning (Experiment 4).

When brain-injured mice ($n = 8$) are weakly conditioned and tested seven days post-injury, fear-associated freezing to the context and cues does not differ from sham ($n = 10$) performance (Fig. 4A; Exp. 3). Freezing behavior during the conditioning was not significantly different between the groups (sham, $11.9\% \pm 5.0$; FPI, $14.3\% \pm 4.5$). Fear-associated activity suppression is evident in both groups of mice to the conditioned context. Therefore, lateral FPI does not affect acquisition or recall of the task on the same day, one week post-injury. Impaired consolidation of the learned association between conditioning on day five and testing on day seven in the weak association paradigm may underlie the cognitive deficit at one week post-injury (Fig. 3; Exp. 2), since mice trained and tested on the same day demonstrate a 21.2% reduction in activity suppression compared to those tested 48 hours later ($p = 0.0976$). In addition, performance during the cue test is similar in brain-injured mice compared to sham, indicating patent acquisition and recall. Moreover, the shortened training and testing timeframe did not generalize to the novel context, as the activity suppression ratios for brain-injured and sham groups are similar and not different from the context or cue groups.

Additionally, mice were conditioned with the strong association paradigm five days after injury and tested 48 hours later (Exp. 4). Freezing behavior during the conditioning was not significantly different between the groups (sham, $11.8\% \pm 8.8$; FPI, $13.8\% \pm 6.8$). Brain-injured ($n = 9$) and sham ($n = 10$) mice exhibited significant decreases in activity suppression ratio for the context and cue compared to the novel context (Fig. 4B). However, no differences were detected between brain-injured and sham groups. Again, the novel context performance indicated no generalization of fear, as values are comparable to preceding groups. In the same time frame, strong association conditioning (Fig. 4B; Exp. 4) masks the acute cognitive deficit observed by weak association training (Fig. 3A; Exp. 2). Therefore, the observed anterograde cognitive deficit at one week after brain injury remains a subtle impairment, as it is overcome by conditioning to more salient stimuli.

Lateral FPI May Remain a Lateralized Hippocampal Injury – Experiment 5

Bilateral aspiration lesions of the hippocampus severely impair cognitive function. However, cognitive recovery has been demonstrated after unilateral hippocampal and entorhinal cortex lesions [39,53], demonstrating that compensatory mechanisms within and across hippocampi reestablish patent cognitive function. To demonstrate that cognitive function can occur with one intact hippocampus, unilateral hippocampal aspiration lesions were performed in uninjured mice one month prior to the conditioned fear task.

At 28 days after unilateral aspiration lesion of the hippocampus ($n = 5$), mice were trained by weak association conditioning and tested 48h later. Freezing behavior during the conditioning was not significantly different between the groups (aspiration, $13.1\% \pm 2.0$; sham, $21.0\% \pm 17.5$; FPI, $22.5\% \pm 21.9$; Exp. 2). The activity suppression ratios for aspiration-lesioned mice in the context (0.23 ± 0.04), cue (0.37 ± 0.04) and novel context (0.45 ± 0.02) were not significantly different from brain-injured or sham-injured mice one month after injury. Hippocampus aspiration resulted in >90% removal of the ipsilateral hippocampus with minimal damage to underlying structures (data not shown). The extent of overlying cortex disruption was not extensive. Within the same time frame, cognitive function evaluated by the conditioned fear paradigm is unaffected one month after unilateral hippocampal aspiration or lateral fluid percussion brain injury, suggesting that a single functional hippocampus may be sufficient for satisfactory cognitive performance.

Behavioral Deficits Predict Neuronal Loss in Hippocampus and No Loss in Amygdala – Experiment 6

The alterations observed in hippocampal-dependent context-dependent conditioned fear at one week after brain injury (Exp. 2) may be due, in part, to neuronal loss in the hippocampus.

Design-based stereology allows for a systematic random sampling of the tissue sections and cells within specific brain regions. Stereology provides a reliable estimation of the actual cell number within the defined region with improved accuracy and precision over profile counting. Quantification of neuronal number using stereology across hippocampal subregions (dentate gyrus, hilus, area CA3 and area CA1) ipsilateral to the injury has demonstrated a uniform and persistent 35-40% loss compared to sham [87,87]. In contrast, neuronal number in the contralateral hippocampus remains unchanged one month after lateral FPI, after a transient decline in neuronal number at one week [82].

Since performance in the conditioned fear paradigm involves the extended limbic system, neuronal estimates were obtained for the ipsilateral and contralateral subiculum of the hippocampus, basolateral complex of the amygdala and central nucleus of the amygdala after lateral FPI. In the present study, the subiculum has been included to complete the anatomical circuit between the hippocampus and the amygdala. Although the estimated number of neurons in each brain region fluctuate between hemispheres (ipsilateral and contralateral) and group (sham [n = 4], 7 day FPI [n = 4] and 28 day FPI [n = 3]), no injury-induced changes in neuronal number were detected (Table 1). Moreover, neuronal number as a percent of sham ranged from 78-102%, verifying preserved neuronal numbers after brain injury. Based on these data, achieving a 95% confidence interval for statistical differences between groups for the ipsilateral hemisphere alone would require 53 animals.

For optimal stereological sampling procedures, the ratio of CE^2/CV^2 should be less than 0.50 [32]. The coefficient of error (CE), measuring intra-animal variation due to the sampling procedure, ranged from 0.07-0.10 (Table 1). The coefficient of variation (CV), which evaluates inter-animal variation due to biological variability, ranged from 0.04-0.39 (Table 1). In the present study, the CE^2/CV^2 ratio is 0.21, verifying a low methodological bias in the precision of estimated neuronal numbers [86].

Discussion

Our study was designed to reproduce a common clinical situation, in which brain-injured patients express varying degrees of anterograde cognitive deficits, which can spontaneously improve, if not completely recover. In the brain-injured mouse, hippocampal- and amygdala-dependent cognitive performance were evaluated in conjunction to determine the extent and nature of remaining cognitive function, using fear-associated freezing in response to conditioned context and cue, respectively. Cognitive deficits after experimental TBI may arise from alterations in acquisition, consolidation or recall, resulting from damage to the hippocampus and/or amygdala [23]. The present data demonstrate an acute injury-induced hippocampal-dependent cognitive deficit in memory consolidation, which can be overcome by more salient stimuli and recovers over time. The absence of neuronal loss from the amygdala suggests that injury-induced damage to the amygdala, if any, may be insufficient to manifest as an amygdala-dependent cognitive deficit in conditioned fear. The face validity with clinical observations of anterograde cognitive deficits highlights the utility of this injury model and demonstrates that not only can cognitive deficits recover, but may be influenced by alternative training paradigms.

Retrograde and anterograde amnesia have been well documented after TBI in humans [30, 52,61]. In the laboratory, the Morris water maze (MWM) and radial arm maze routinely demonstrate injury-related cognitive deficits [37,54,63,67]. However, contextual fear response minimizes the impact of motor deficits and motivation [37,46,66]. Previously, concurrent exposure to the context and footshock has demonstrated a persistent anterograde cognitive deficit after experimental FPI, which may bypass the hippocampus and represent impairments in acquisition, consolidation, and recall [41]. The current training paradigms used a 90 second

interval between the US and the CS, which has been shown to be the maximal temporal pairing necessary to unmask injury-induced deficits and incorporate hippocampal processing [41,62]. For the first time, we evaluated amygdala-dependent conditioning along with the novel context to demonstrate the specificity of the training, rather than generalization across testing conditions.

Unilateral TBI produced a subtle anterograde hippocampal cognitive deficit in consolidation (Exp. 2), which recovered by one month (Exp. 2) to a level commensurate with a unilateral hippocampal aspiration lesion (Exp. 5). The impermanence of the cognitive deficit suggests that unilateral brain injury resembles unilateral hippocampal damage [39,53], more than enduring bilateral dysfunction. The observed cognitive impairment, rather than a complete deficit, may involve acquisition, consolidation or recall. Brain-injured mice trained 48 hours prior to the test are unable to recall conditioned information (Exp. 2) indicating that acquisition on day five and/or consolidation between days five and seven is/are impaired. Brain-injured mice trained acutely prior to the test (Exp. 3) can recall conditioned information, suggesting that cognitive functions of acquisition and recall remain intact (or have recovered) on day 7 post-injury. Since mice trained using the strong association on day five and tested 48 hours later retain the conditioned associations (Exp. 4), the neural circuitry responsible for the conditioned association remains intact. Taken together, these data indicate that a deficit in the consolidation of the weak conditioned associations may predominate after mild/moderate FPI. Alternatively, acquisition of the weak association may be impaired up to day five post-injury, with the possibility of recovery occurring over the ensuing two days. In a retrograde amnesia paradigm after concussion brain injury, specific cognitive deficits in retrieval, rather than memory consolidation or storage, have been demonstrated [89], which may or may not resemble anterograde cognitive dysfunction. The inclusion of protein synthesis inhibitors before, during and after training could further elucidate the nature of the hippocampal-dependent consolidation deficit [49,50,74]. In the amygdala, the absence of neuronal loss substantiates the absence of an amygdala-dependent cognitive deficit, leaving the conditioned context deficit likely to result from functional alterations in remaining injured hippocampal neurons [88]. One day after brain injury, glucose utilization during spatial learning is elevated significantly in the ipsilateral amygdala and reduced significantly in the hippocampus compared to sham values, demonstrating injury-induced alterations in limbic information processing [70]. In fact, transient alterations in circuit or synapse function at one week may be restored by one month, to mediate the recovery of cognitive function [82]. More severe lateral brain injuries (longer righting reflex times or greater mortality) may produce more profound and persistent cognitive deficits. Under the present injury parameters, however, the pathology remains predominantly in the ipsilateral hippocampus [82,88].

In the single trial anterograde training, extinction is not reported to play a role. Extinction occurs for the context in which animals are repeatedly tested, without retraining [72]. Brain-injured and sham-injured animals were exposed to each testing environment once. Furthermore, uninjured animals demonstrate specific fear-related responses to conditioned parameters, but not the novel context. Additionally, the nature of the behavioral evaluations do not warrant changes in neuronal number.

To allow the initial pathological TBI cascades to subside [81], fear-associated freezing was assessed at time points after injury. In addition, the delayed training-testing interval of 48 hours evaluates long-term memory consolidation and recall, for which synaptic plasticity, receptor activation and protein synthesis are required [1]. However, TBI-induced alterations in plasticity [5,26,43], protein synthesis [44,68], and glucocorticoid levels [34,35] could interfere with delayed cognitive performance, requiring shorter time intervals to evaluate gene expression- and protein synthesis-independent conditioned fear [49,71]. Nonetheless, the present brain injury model involves an anterograde cognitive deficit and recovery (Exp. 2), which can be

exploited to isolate mechanisms responsible for the recovery observed after human TBI. Clinically, cognitive function can improve after brain injury, despite reductions in brain volume [27].

The extent of hippocampal dependence in the contextual conditioning of fear remains unknown. Task performance is hippocampal-dependent in the time-limited acute period surrounding training, where relevant hippocampal lesions or ablations disrupt the associative conditioning. In contrast, hippocampal lesions made four weeks after training are ineffective in abolishing the learned associations [8]. Yet, the hippocampal-dependence of conditioned fear has not been evaluated in the injured brain. In the current data set, animals were tested two days after training, where the hippocampus retains a role in the task. Moreover, a single feature of the context (such as the type of floor), rather than the context itself, may become the conditioned stimulus after brain injury. In this way, animals would switch from a hippocampal-dependent configural association strategy to a hippocampal-independent elemental association strategy to perform in the task [58]. However, the performance differences in the context, cue and novel context negate alternative learning strategies during training.

Despite prior reports of injury-induced pathophysiology in the amygdala [2,19], amygdala-dependent cue conditioned deficits could not be detected after lateral FPI in the mouse. By four weeks post-injury, quantification of principal neurons in the amygdala basolateral complex are suggestive of a delayed degeneration (Exp. 6), where cognitive deficits may be revealed at later time points. Conditioned fear to distinct, prominent cues may be more resilient to injury than contextual cues [4,13]. In the strong conditioning paradigm (Exp. 4), the light stimulus may be responsible for strengthening the conditioning to the cue, especially since compromised ear drums from the stereotaxic frame may reduce auditory thresholds for the tone. Similarly, the lateralization of the injury and resulting pathology may not sufficiently disrupt bilateral circuit function to elicit global cognitive deficits. Blair et al. (2005) recently demonstrated unilateral conditioning of amygdala-dependent fear associations, which may be more sensitive for future detection of lateralized injury-induced dysfunction [14].

The unique consequences of a unilateral brain injury include a global cognitive deficit, not normally observed after standard unilateral hippocampal lesions [39,53]. After lateral TBI, disrupted cognitive function is concomitant with ipsilateral neuronal death and bilateral circuit disruption, which define the post-injury stunned brain. Acute perturbations of glial function and ionic fluxes highlight the transient nature of injury-induced alterations that can alter excitability [21,45]. Lastly, disruption of synapse function or number [77,80], without neuronal loss, could contribute to the acute injury-induced deficits in conditioned fear, prompting future studies at the ultrastructural level. It follows, therefore, that the natural course of functional recovery in experimental and clinical TBI may be amenable to therapeutic intervention to overcome the transient lesion.

Brain injury disrupts cognitive function at one week, but cellular and molecular plasticity appear to permit recovery over the first month. With this knowledge, future studies can be directed towards the mechanisms underlying the recovery of cognitive function in an injured, but not ablated, brain. The underlying anatomical or functional alterations that contribute not only to the injury-induced cognitive impairment but the subsequent recovery in experimental TBI remain to be determined.

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Figure 1.

Schematic representations of the conditioning (training) protocols, testing protocols, and the calculation of the activity suppression ratio. The time course of the conditioning paradigms is outlined below each heading, with separations for each minute. At the appropriate time(s), both the conditioning stimuli (light grey above the line) and the foot shock (medium grey above the line) are indicated. Similarly, the time course for testing 48 hours after conditioning is illustrated. Below the line, periods of activity that are included in the calculation of the activity suppression ratio (dark grey, A, B₁, B₂, B₃) are indicated, where B₁, B₂, B₃ give context, cue and novel context ratios, respectively.

Figure 2.

Evaluation of the weak and strong association conditioning paradigms in naïve mice (Experiment 1). (A) According to the schematic timeline, mice were handled, classically conditioned to associate the training context and cues (light and/or sound) with a foot shock, and tested for fear-associated freezing to the context or cues. Recall of the conditioned associations were measured as the percent fear-associated freezing in each minute over the five minute test. (B) In the trained context, hippocampal-dependent recall of the contextual conditioning is demonstrated as elevated and stable freezing. (C) In the novel context, the absence of generalized fear is demonstrated in the first two minutes before the conditioned cues are activated for the remaining time and show amygdala-dependent recall of the

conditioning. (D) The activity suppression ratio indicates the extent of fear (0.0 = fear; 0.5 = no fear) normalized to each animal's baseline activity. †, $p < 0.05$ compared to context; *, $p < 0.05$ compared to weak association.

Figure 3.

By varying the temporal spacing of the conditioning and testing with respect to brain injury, the effects of lateral FPI on cognitive function were evaluated. Anterograde cognitive function was evaluated one week (A) and one month (B) after brain injury according to the schematic timeline (Experiment 2). Activity suppression ratio data are given for the context, cue and novel context in sham and brain-injured (FPI) mice. †, $p < 0.05$ compared to context; *, $p < 0.05$ compared to sham.

Figure 4.

To determine the nature of the cognitive deficit, uninjured and brain-injured mice were trained and tested on the same day (A, Experiment 3) or conditioned using the strong association paradigm at one week after injury (B, Experiment 4). No statistically significant differences were detected between cue and context or brain-injured (FPI) and sham mice.

Table 1

Estimated total number of Nissl-stained neurons in the subiculum, basolateral complex of the amygdala and central nucleus of the amygdala that are ipsilateral and contralateral to sham or brain injury (FPI).

	N ±SD	ΣQ̄ ±SD	Mean CE	CV	% Sham
Subiculum					
Sham					
Ipsilateral	152,882.3 ±16,895.1	405.3 ±42.5	0.10	0.11	103%
Contralateral	143,148.8 ±12,275.6	376.0 ±13.6	0.10	0.09	97%,
7d FPI					
Ipsilateral	130,637.3 ±35,062.0	348.3 ±100.8	0.09	0.27	88%
Contralateral	140,646.5 ±26,193.0	373.3 ±81.9	0.10	0.19	95%
30d FPI					
Ipsilateral	119,924.0 ±15,722.3	325.0 ±42.8	0.10	0.13	81%
Contralateral	150,398.8 ±27,337.7	407.7 ±74.3	0.08	0.18	102%
Basolateral Complex					
Sham					
Ipsilateral	100,219.1 ±8,342.8	275.3 ±24.9	0.09	0.08	99%
Contralateral	102,752.0 ±12,242.2	281.8 ±35.8	0.07	0.12	101%
7d FPI					
Ipsilateral	101,096.3 ±26,820.9	276.0 ±80.1	0.08	0.27	100%
Contralateral	104,998.8 ±18,227.4	283.8 ±58.9	0.08	0.17	103%
30d FPI					
Ipsilateral	84,508.3 ±3,312.0	233.3 ±8.7	0.08	0.04	83%
Contralateral	88,813.9 ±9,212.8	245.3 ±25.1	0.07	0.10	88%
Central Nucleus					
Sham					
Ipsilateral	29,018.2 ±6,260.1	244.5 ±52.3	0.08	0.22	97%
Contralateral	30,901.6 ±9,035.2	260.5 ±77.1	0.10	0.29	103%
7d FPI					
Ipsilateral	27,530.5 ±10,763.7	229.0 ±95.5	0.08	0.39	92%
Contralateral	38,672.3 ±10,119.4	320.8 ±92.2	0.10	0.26	129%
30d FPI					
Ipsilateral	26,407.8 ±4,410.7	223.3 ±38.0	0.09	0.17	88%
Contralateral	28,549.3 ±9,515.5	241.7 ±81.0	0.08	0.33	95%

Data are group means ± standard deviation. CE = coefficient of error, CV = coefficient of variation