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Old Dog, New Tricks: The Attentional Set-Shifting Test as a Novel Cognitive Behavioral Task after Controlled Cortical Impact Injury

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Abstract

Cognitive impairment associated with prefrontal cortical dysfunction is a major component of disability in traumatic brain injury (TBI) survivors. Specifically, deficits of cognitive flexibility and attentional set-shifting are present across all levels of injury severity. Though alterations in spatial learning have been extensively described in experimental models of TBI, studies investigating more complex cognitive deficits are relatively scarce. Hence, the aim of this preclinical study was to expand on this important issue by evaluating the effect of three injury levels on executive function and behavioral flexibility performance as assessed using an attentional set-shifting test (AST). Isoflurane-anesthetized male rats received a controlled cortical impact (CCI) injury (2.6, 2.8, and 3.0 mm cortical depth at 4 m/sec) or sham injury, whereas an additional group had no surgical manipulation (naïve). Four weeks postsurgery, rats were tested on the AST, which involved a series of discriminative tasks of increasing difficulty, such as simple and compound discriminations, stimulus reversals, and intra- and extradimensional (ED) shifts. TBI produced accompanying impact depth-dependent increases in cortical lesion volumes, with the 3.0-mm cortical depth group displaying significantly larger injury volumes than the 2.6-mm group ($p = 0.05$). Further, injury severity-induced deficits in ED set-shifting and stimulus reversals, as well as increases in total response error rates and total set loss errors, were observed. These novel findings demonstrate executive function and behavioral flexibility deficits in our animal model of CCI injury and provide the impetus to integrate the AST in the standard neurotrauma behavioral battery to further evaluate cognitive dysfunction after TBI. Ongoing experiments in our laboratory are assessing AST performance after pharmacological and rehabilitative therapies post-TBI, as well as elucidating possible mechanisms underlying the observed neuropsychological deficits.

Key words: attentional set-shifting; behavior; controlled cortical impact; executive function; traumatic brain injury

Introduction

Approximately 1.5 million Americans suffer a traumatic brain injury (TBI) each year, with cognitive impairments being the hallmark of long-term disability.[1–3] TBI survivors exhibit a multitude of long-lasting cognitive sequelae that have been associated with reduced quality of life and work productivity,[4,5] as well as mental and emotional disturbance comorbidities.[6] Observed cognitive deficits typically include alterations in learning, memory, and higher-order executive functions, consisting of attention and memory loss, inability to acquire or store new information,[7] and impairments in planning and behavioral flexibility.[8] A substantial experimental body of TBI literature employing fluid percussion, controlled cortical impact (CCI), or blast injury approaches has associated brain injury with declines in numerous cognitive processes, such as reference memory on the Morris water maze (MWM) test,[9–13] working memory in the Barnes maze test,[14,15] or a combination of both in the radial arm maze test,[16,17] as well as declarative memory in the novel object recognition test.[18,19] However, studies investigating more complex cognitive deficits are relatively scarce, and, in order to more accurately mimic injury-related cognitive symptoms in humans, with the ultimate goal for more accurate means of treatment and rehabilitation, it is important to further evaluate TBI-induced alterations in frontal lobe-mediated attentional processes.

Executive function and behavioral flexibility represent advanced capabilities to ''unlearn'' an established contingency in order to acquire a new one by shifting attention from a salient stimulus dimension to a previously irrelevant one.[20] In humans, the Wisconsin Card Sorting Test (WCST) has been successfully employed

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as a well-validated neuropsychological measure of complex cognitive processes and was shown to sensitively characterize strategy-switching impairments in patients with TBI,[21–24] depression, and other neuropsychiatric disorders thought to affect the prefrontal cortex.[25–27] To specifically explore the different facets of behavioral flexibility assessed in the WCST, Birrell and Brown developed an analogous behavioral test to measure attentional set-shifting performance in rats and demonstrated a pivotal role played by the rodent prefrontal cortex (PFC) in mediating complex cognitive processes in this test, such as attentional set-shifting[28] and stimulus reversal learning.[29] The attentional set-shifting test (AST) comprises a series of increasingly difficult perceptual discriminations to obtain a food reward that requires subjects to form and maintain an attentional set or subsequently shift from one stimulus dimension to another that previously served as an overlapping distractor.[20,28,30] In this test, rats are trained to retrieve a food reward from small terracotta pots containing distinctive digging media and marked with different odors on the upper rim. Subjects must learn to recognize the salient stimulus dimension (odor or digging medium) associated with the reward, based on a predetermined randomized order of stimulus presentation and overlying secondary dimension distractors, while facing rule changes as in the WCST upon learning a given contingency.[20] Cognitive inflexibility and perseveration in the AST have been extensively described in rodent models of various neuropsychiatric and emotional disorders, such as in the spontaneously hypertensive rat, a genetic model of attention-deficit hyperactivity disorder,[31] the neonatal ventral hippocampal lesion model,[32] the 6-hydroxydopamine model of Parkinson's disease,[33] in glutamate N-methyl-D-aspartate receptor antagonist-induced schizophrenia-like symptomatology,[34–36] cognitive decline during aging,[37] and chronic unpredictable[20,38] or restraint stress[39] exposure. Therefore, in order to begin to address putative alterations in frontal cortex-mediated EF in a rodent model of TBI, we set out to determine whether a CCI injury of various cortical deformation depths would induce impact depth-dependent cognitive deficits in the attentional performance of adult male Sprague-Dawley rats on the AST. These results could provide the impetus to integrate the AST in the standard neurotrauma behavioral battery after TBI in order to evaluate sensitive cognitive dysfunction and subsequent relevant therapies.

Methods

Animals

A total of 47 adult male Sprague-Dawley rats (Harlan Laboratories Inc., Indianapolis, IN) were housed in commercially available opaque polyethylene cages and maintained in a temperature-(21 ± 1°C) and light-controlled (12/12-h light/dark cycle; lights on at 7:00 AM) environment with food and water available *ad libitum*. After 1 week of acclimatization to the housing facility, the experimental manipulations began and were carried out during the light portion of the cycle. All procedures were conducted in accord with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 2010) and were reviewed and approved by the institutional animal care and use committee at the University of Pittsburgh (Pittsburgh, PA). All efforts were made to minimize animal pain, suffering, or discomfort and to minimize the number of rats used.

Surgery

On the day of surgery, rats weighing 300–325 g were randomly assigned to CCI and sham injury groups or had no surgical manipulation (naïve). All surgical procedures were performed as previously described.[10,11,13,40–44] Briefly, isoflurane anesthesia was induced and maintained using a 2:1 N_2O/O_2 gas mix at concentrations of 4% and 2%, respectively. Rats were subsequently intubated endotracheally, placed on mechanical ventilation for anesthesia maintenance, and secured in a stereotaxic frame. A heating pad was used to maintain core temperature at 37 ± 0.5°C, which was measured with a rectal probe throughout surgery. Using aseptic procedures, a craniectomy was performed in the right hemisphere with a dental drill. A TBI was then produced by impacting the exposed right parietal cortex to a depth of 2.6, 2.8, and 3.0 mm tissue deformation at 4 m/sec. After the impact, anesthesia was discontinued and the incision was promptly sutured. Sham injury rats were not subjected to the cortical impact, but otherwise underwent similar surgical procedures, whereas naïve rats received no surgical manipulations.

Acute neurological evaluation

Subsequent to cessation of anesthesia, hindlimb reflexive ability was determined by briefly squeezing the rats' paw every 5 sec and recording the time to elicit a withdrawal response. Return of the righting reflex also was assessed by recording the time required to turn from the supine to prone position. Upon return to the housing facility, rats were then weighed daily until the day of testing.

Attentional set-shifting test

Procedures for the AST were as described previously,[30,38] adapted from Birrell and Brown.[28] One week preceding behavioral testing (i.e., on day 21 postsurgery), rat food intake was limited to 14 g/day, with water freely available. To ensure that injured rats were not more susceptible to losing weight during the food restriction period, compared to control groups, weight lost was measured on testing day as a percentage of body weight at initiation of food restriction for each animal. The testing apparatus was a custom-built rectangular Plexiglas arena (width × length × height, $30 \times 51 \times 25$ cm), painted gray on the outer side of all surfaces (Fig. 1A). A removable divider separated one third of the arena forming a start box, which was also used as a holding area between trials to allow the experimenter to clean the arena and change the pots. To begin each trial, the rat was given access to the arena by lifting the divider. A clear Plexiglas panel divided the opposite third of the

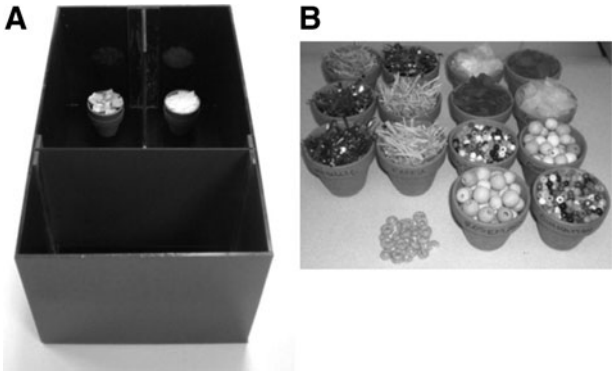

FIG. 1. (**A**) The attentional set-shifting Plexiglas apparatus, fitted with a removable divider separating the start box, which serves as a holding area between trials, and the digging terracotta pots defined by a pair of cues along two stimulus dimensions (an odor and the digging medium filling the pot; pictured in the arena are pots for the simple discrimination training session for medium: paper strips and felt pieces). (**B**) Digging terracotta pots illustrating stimulus pairs used throughout the attentional set-shifting test stages and the Honey Nut Cheerios reward (General Mills Cereals, Minneapolis, MN).

arena into two sections, with one digging pot in each section. This separation enabled quick removal of the rat after a response. The terracotta digging pots (internal rim diameter, 7 cm; depth, 6 cm) were each defined by a pair of cues along two stimulus dimensions: the digging medium filling the pot and an odor (Fig. 1B; Table 1). To mark each pot with a distinct odor, two drops (10 μL/drop) of scented aromatic oil (NOW Foods, Bloomingdale, IL) were applied to the inner rim at least 5 days before initial use. Then, 1–2 μL were reapplied daily to maintain consistent odor intensity. A different pot was used for each combination of digging medium and odor, and only one odor was ever applied to a given pot. The bait, a one-quarter Honey Nut Cheerio (General Mills Cereals, Minneapolis, MN), was buried 2 cm below the surface of the digging medium in the ''positive'' pot. A small quantity of powdered Cheerio dust was sprinkled over the digging media in both pots before beginning each task to prevent the rat from locating the bait by smell rather than by learning the discrimination.

Digging was defined as a vigorous displacement of the medium to retrieve the reward buried within the pot. Simply investigating the rim of the pot or the surface of the digging medium with paws or snout without displacing material was not considered a ''dig'' and the trial continued until a dig response was performed. Therefore, rats were able to access tactile, visual, and olfactory characteristics of the pots to make their choices. The behavioral procedure comprised 3 consecutive days.

Habituation (day 1). Rats were first trained to dig reliably in pots to obtain a food reward. Two unscented pots were placed in the home cage and rebaited every 5 min, with the Cheerio covered by increasing amounts of sawdust (three times with no sawdust, three times with the pots one-third full, three times half full, and three times completely full). Once the rat was digging reliably, it was transferred to the testing arena and given three trials to retrieve the reward from both sawdust-filled pots.

Training (day 2). Rats were trained on a series of simple discriminations (SD) in order to reach a criterion of six consecutive correct trials. First, they learned to associate the food reward with an odor cue (lemon vs. eucalyptus, both pots filled with sawdust). After reaching criterion for the odor discrimination, rats had to learn to discriminate between digging media in new unscented pots (felt vs. paper strips). All rats were trained using the same pairs of stimuli and in the same order. The positive and negative cues for each rat were randomly determined and equally represented. The training exemplars were not used again during testing. Any rat that failed to complete the training procedure was eliminated from subsequent testing.

Testing (day 3). Rats were then tested on a series of increasingly difficult discrimination stages (Table 1), each requiring a criterion of six consecutive correct trials. At each testing stage, the discriminative stimulus dimension and the positive cue within that dimension were varied according to the cue progression shown in Table 1. The first stage was an SD involving only one stimulus dimension (i.e., odor or medium). Half the animals in each group were required to discriminate between sawdust-filled pots differentiated by two odors, only one of which was associated with reward. For the other half, the first discrimination was between digging media in unscented pots (for clarity, the following description will consider only the example beginning with an odor discrimination). The second stage was a compound discrimination (CD), in which the same contingency rule was required (e.g., odor), and the second, irrelevant stimulus dimension (e.g., medium) was introduced. As in the SD task, only one odor was associated with reward, but two different digging media were paired randomly with the odors. The third stage was a reversal (R1) of the previous discrimination, in which the same odors and media were used. Odor was maintained as the relevant dimension; however, the negative odor from the previous stage became positive (i.e., was associated with reward), and the positive odor from the previous stage became negative (no reward). The fourth stage was an intradimensional (ID) shift in which all new stimuli (odors and media) were introduced. Again, odor remained the relevant dimension and digging medium was still irrelevant. The fifth stage was a reversal of this discrimination (R2), in which the previously negative odor became positive, similar to R1. The sixth task required an extradimensional (ED) cognitive set-shift, in which all new stimuli were again introduced, but the dimension that had been repeatedly reinforced as the informative, relevant dimension (thus forming a ''cognitive set'') was now irrelevant, and the previously distracting dimension (i.e., the digging medium) became the relevant dimension. Finally, the seventh stage was another reversal (R3), where the previously negative stimulus became positive, as in the previous reversals. The assignment of each exemplar in a pair as being positive or negative

Table 1. Representative Examples of Stimulus Pairs and the Progression Through the AST Stages

	Dimensions		*Stimulus combinations*	
Test day: odor discrimination stage	*Relevant*	*Irrelevant*	*(+)*	*(−)*
Simple (SD)	Odor		**Clove**/sawdust	Jasmine/sawdust
Compound (CD)	Odor	Medium	**Clove**/raffia	Jasmine/metal confetti
			Clove/metal confetti	Jasmine/raffia
Reversal (R1)	Odor	Medium	**Jasmine**/metal confetti	Clove/raffia
			Jasmine/raffia	Clove/metal confetti
Intradimensional shift (ID)	Odor	Medium	**Rosemary**/wood balls	Cinnamon/plastic beads
			Rosemary/plastic beads	Cinnamon/wood balls
Reversal (R2)	Odor	Medium	**Cinnamon**/plastic beads	Rosemary/wood balls
			Cinnamon/wood balls	Rosemary/plastic beads
Extradimensional shift (ED)	Medium	Odor	**Velvet**/lavender	Crepe/peppermint
			Velvet/peppermint	Crepe/lavender
Reversal (R3)	Medium	Odor	**Crepe**/peppermint	Velvet/lavender
			Crepe/lavender	Velvet/peppermint

This example portrays odor as the initial discriminative dimension, followed by a shift to digging medium in the ED stage. For each stage, the positive stimulus is in bold and is paired randomly across trials with the two stimuli from the irrelevant dimension.

AST, attentional set-shifting test.

in a given stage, as well as the left-right positioning of the pots in each trial, were determined randomly in advance. The dependent measure was the number of trials required to reach criterion of six consecutive correct responses (trials to criterion) for each stage of the test. Animals were allowed 10 min to make a choice on each trial. If a choice was not made within this interval, the trial was scored as an error and the rat was returned to the start box. Animals failing to make a choice on six consecutive trials, or failing to complete a stage within 50 trials, were eliminated from further testing.

Histology: Cortical lesion volume

After completion of behavioral testing (i.e., 5 weeks post surgery), rats were anesthetized with Fatal-Plus® (Henry Schein Animal Health, Columbus, OH 0.25 mL, intraperitoneally) and then perfused transcardially with 200 mL of 0.1 M of phosphate-buffered saline (pH 7.4), followed by 300 mL of 4% paraformaldehyde (PFA). Brains were extracted, postfixed in 4% PFA for 1 week, dehydrated with alcohols, and embedded in paraffin. Seven-micrometer-thick coronal sections were cut at 1-mm intervals through the lesion on a rotary microtome and mounted on Superfrost®/Plus glass microscope slides (Fisher Scientific, Pittsburgh, PA). After drying at room temperature, sections were deparaffinized in xylenes, rehydrated, and stained with cresyl violet. Cortical lesion volumes (mm^3) were assessed by an observer blinded to experimental conditions using a Nikon Eclipse 90i microscope (Nikon Corporation, Tokyo, Japan). The area of the lesion (mm^2) was first calculated by outlining the inferred area of missing cortical tissue for each section (typically 5–7; Nikon NIS-Elements AR 3.22.14 software; Nikon) and then by summing the lesions obtained, as previously reported.[45–48]

Statistical analyses

Statistical analyses were performed on data collected by observers blinded to treatment conditions using StatView 5.0.1 software (Abacus Concepts, Inc., Berkeley, CA). Righting reflex, hindlimb reflexive ability, and weight loss were analyzed by one-way analysis of variance (ANOVA), whereas histological data were analyzed using unpaired *t*-tests. For analysis of behavior on the AST, the total trials to criterion, total response errors, and set loss errors were recorded for each test stage. These data were analyzed by two-way ANOVA (group × stage), with repeated measures over stage. Where significant, ANOVA main effects or interactions were indicated, and post-hoc comparisons to determine specific group differences on individual stages were performed using Newman-Keuls test or post-hoc one-way ANOVAs, as appropriate. Results are expressed as the mean ± standard error of the mean (SEM), and significance for all analyses was determined at $p < 0.05$.

Results

A total of 8 rats were eliminated and were not included in the statistical analyses. Three of these animals died after surgery (1 in each injury group), 2 rats were eliminated because they did not complete the training procedure (all subjected to injury), and 3 more did not complete the behavioral test procedure (all injured rats).

Acute neurological evaluation and body weights

There were no significant differences among TBI groups with respect to hindlimb reflex withdrawal latency in response to a brief paw pinch administered to either limb (left range = 159.3 ± 7.6 to 169.8 ± 6.8 sec, $p > 0.05$; right range = 155.1 ± 7.5 sec to 165.7 ± 7.0 sec, $p > 0.05$) after termination of anesthesia. However, ANOVA detected significant differences among TBI groups for return to righting ability ($F_{3,29} = 40.958$, $p < 0.0001$; TBI [2.6 mm], 343.8 ± 12.7 sec; TBI [2.8 mm], 370.1 ± 17.2 sec; TBI [3.0 mm], 420.8 ± 28.4 sec). Post-hoc comparisons revealed an increase in return to righting ability in all TBI groups, compared to the sham group ($p < 0.05$), as well as an injury impact-dependent increase in righting

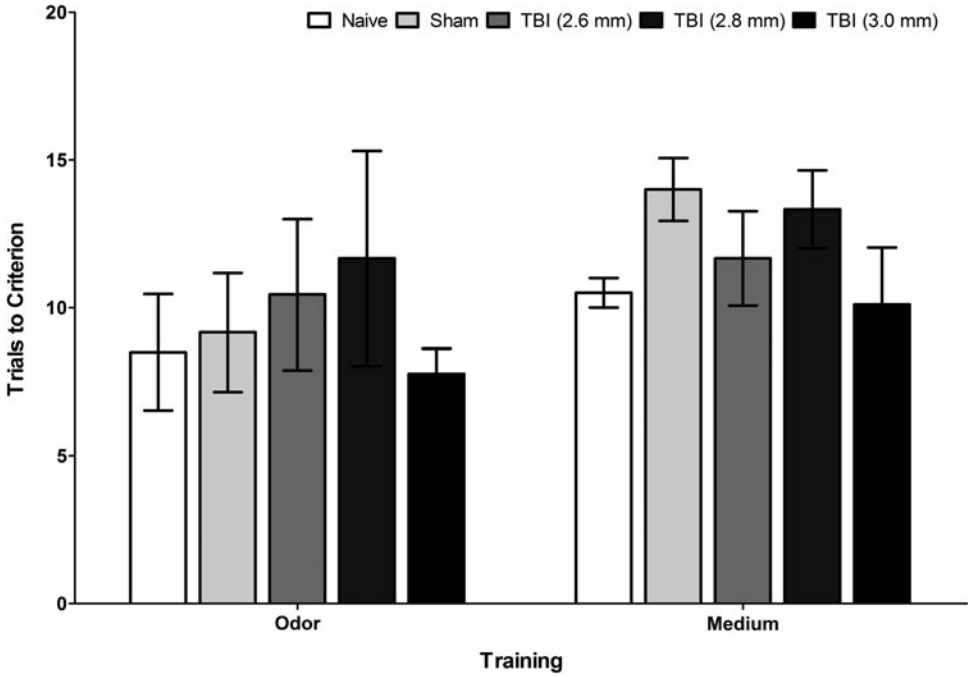

FIG. 2. Mean (± standard error of the mean [SEM]) trials to reach criterion on simple discriminations tasks on the training day preceding testing. There were no significant differences among groups, whether subjected to TBI injury (TBI [2.6 mm], TBI [2.8 mm], and TBI [3.0 mm]) or controls (naïve and sham groups), on ability to complete training procedures with relevant dimension being either odor or medium. Data are expressed as mean trials to criterion ± SEM ($n = 6$–9/group; $p > 0.05$).

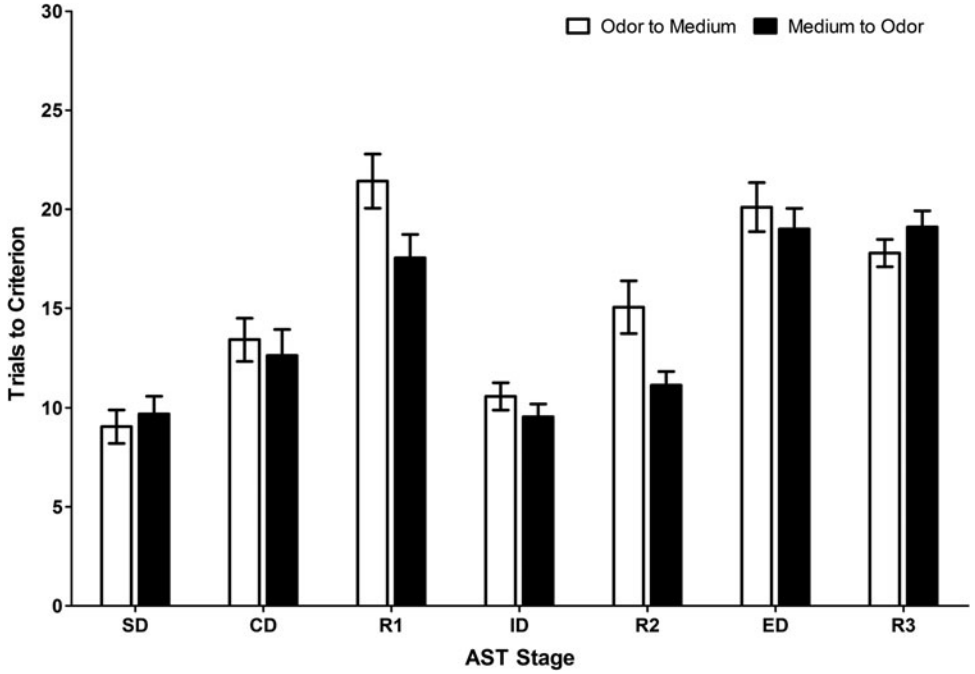

FIG. 3. Progression through the attentional set-shifting protocol stages renders comparable cognitive performance patterns regardless of the initial discriminative dimension (odor or medium), suggesting no injury-related bias against a sensory modality necessary for contingency rule acquisition. Data are expressed as mean trials to criterion ± standard error of the mean ($n = 19$–20/group). SD, simple discriminations; CD, compound discrimination; ID, intradimensional; ED, extradimensional.

ability with a statistically significant difference between the TBI (3.0 mm) and TBI (2.6 mm) groups ($p < 0.05$).

A one-way ANOVA assessing body-weight loss during the 1 week of mild food restriction of 14 g/day for each rat up to the testing day revealed no differences among groups, regardless of whether they were control or injured rats ($F_{4,34} = 1.37$; $p = 0.265$). Therefore, injured rats were not more susceptible to losing weight during the food restriction process when initiated at 3 weeks postsurgery.

Attentional set-shifting test

There was no significant difference among groups for the number of trials required to reach criterion for the SD task on the training day ($F_{4,\ 34} = 0.\ 843$; $p = 0.508$; Fig. 2). Figure 3 shows a comparison between rats subjected to an odor to medium versus medium to odor ED set-shift on the trials to reach the criterion for each task of the AST regardless of group. There were no significant differences in cognitive performance on the AST between ED shift-type designations ($F_{1,\ 37} = 3.201$; $p = 0.082$; Fig. 3), suggesting that injury exposure did not bias rats to perform differentially depending on whether the contingency belonged to a certain dimension, such as odor or medium.

Figure 4 shows the effects of CCI injury on the trial to criterion for each stage of the AST at 4 weeks postsurgery. A two-way ANOVA (group × stage), with repeated measures over stage, revealed significant main effects of group ($F_{4,\ 34} = 5.744$; $p < 0.005$) and stage ($F_{6,\ 204} = 37.641$; $p < 0.0001$), but no group x stage interaction ($F_{24,\ 204} = 1.397$; $p = 0.111$). For the main effect of stage, post-hoc comparisons across all groups showed that significantly more trials were required to reach criterion during R1, R3, and ED than in other stages (Fig. 4). As previously reported, this effect validates the inherent difficulty of the stimulus reversal and ED set-shifting stages, compared to the other stages of the AST.[28,30,38] Further, post-hoc comparisons for the group effect revealed that CCI injury induced overall significant impact depth-dependent performance deficits. Specifically, rats in the TBI (2.8 mm) and TBI (3.0 mm) deformation depth groups displayed significant cognitive performance impairments, manifested as an increase in trials to criterion, relative to naïve and sham control groups as well as the TBI (2.6 mm) group (all $p < 0.05$). Subsequent post-hoc one-way ANOVAs for individual AST stages revealed significant injury effects on the ED set-shifting stage ($F_{4,\ 34} = 5.834$; $p < 0.005$) and the third reversal (R3; $F_{4,\ 34} = 3.362$; $p < 0.05$). Post-hoc analyses using Newman-Keuls test also determined that on the ED stage, rats in the TBI (2.8 mm) and TBI (3.0 mm) groups performed significantly worse than the naïve and sham control groups ($p < 0.05$), as well as the mild TBI group (TBI [2.6 mm]; $p < 0.05$; Fig. 4), because they required a higher number of trials to reach the criterion on this stage. Similarly, post-hoc analyses for individual group comparisons on the R3 stage revealed a significant injury effect for the TBI (2.8 mm) group, relative to the sham group, observed as increased trials to criterion in this stimulus reversal task ($p < 0.05$; Fig. 4). No other main effects or interactions were revealed on any other test stages.

With respect to total error responses on the AST, ANOVA revealed a significant effect of group ($F_{4,\ 34} = 2.\ 585$; $p = 0.05$) and stage ($F_{6,\ 204} = 35.932$; $p < 0.0001$), but no significant group x stage interaction ($F_{24,\ 204} = 1.352$; $p = 0.134$; Fig. 5). Specifically, Newman-Keuls post-hoc comparisons for overall group effects did not reveal significant differences between injury and control groups across all AST stages ($p > 0.05$), but showed a significant individual-stage group effect on R1 ($F_{4,\ 34} = 2.759$; $p < 0.05$), with TBI rats displaying higher rates of total response errors while learning

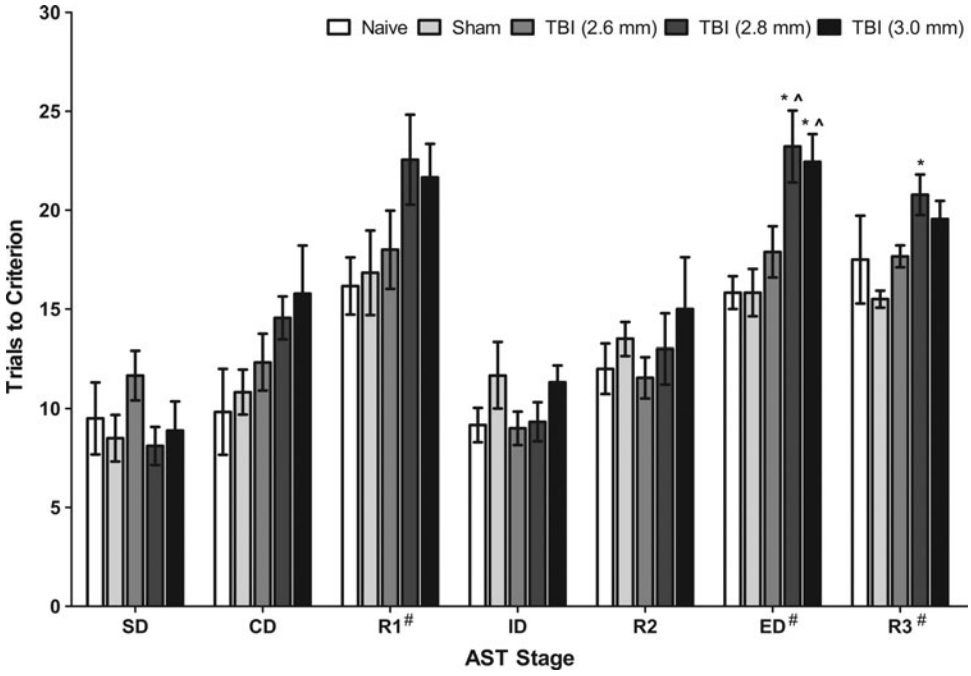

FIG. 4. Impact depth-dependent effect of controlled cortical impact (CCI) injury, ranging from 2.6 to 3.0 mm cortical deformation depth, on cognitive performance on the AST. Significantly more trials were required to reach criterion on the R1, ED, and R3 tasks (main effect of stage, $^{\#}p < 0.05$, compared to the other stages, collapsed across surgical groups). CCI injury produced impairments in cognitive performance on the AST, manifested as an overall significantly higher number of trials to criterion across AST stages for both the TBI (2.8 mm) and TBI (3.0 mm) groups, compared to naïve, sham, and TBI (2.6 mm) deformation depth groups (all $p < 0.05$). Newman-Keuls' post-hoc analyses for individual test stages determined that the CCI injury also induced significant performance deficits in the moderate and severe injury groups (2.8 and 3.0 mm, respectively), relative to naïve, sham ($^{*}p < 0.05$), and TBI (2.6 mm) groups ($^{\wedge}p < 0.05$) on the ED stage, as well as on the third reversal stage in the TBI (2.8 mm) group, compared to sham controls ($^{*}p < 0.05$). Data are expressed as mean trials to criterion ± standard error of the mean ($n = 6$–9/group). TBI, traumatic brain injury; SD, simple discriminations; CD, compound discrimination; ID, intradimensional; ED, extradimensional; AST, attentional set-shifting test; R1, first reversal; R2, second reversal; R3, third reversal.

the contingency rule toward reaching criterion (Fig. 5). Subsequent post-hoc analyses revealed significant performance impairment in the TBI (2.8 mm) group, compared to the naïve group, observed as increased total errors on the first reversal (R1) stage ($p < 0.05$; Fig. 5). No other main effects or interactions were revealed on any other test stages.

Set loss errors for each rat on every stage were calculated as the errors occurring after 50% or more of contingency acquisition has been achieved (i.e., an error after three, four, or five correct responses), parallel to the WCST.[26] Repeated-measures ANOVA found significant effects of group ($F_{4,\ 34} = 6.805$; $p < 0.001$) and stage ($F_{6,\ 204} = 5.571$; $p < 0.0001$), however not a group x stage interaction ($F_{24,\ 204} = 1.400$; $p = 0.101$). Post-hoc analyses for the group effect determined that rats in the TBI (2.8 mm) cortical deformation depth group displayed significantly higher set loss errors throughout the AST stages, when compared to the naïve and TBI (2.6 mm) groups, whereas rats in the TBI (3.0 mm) group displayed significantly higher overall set loss errors, relative to both naïve and sham controls as well as the TBI (2.6 mm) group (all $p < 0.05$; Table 2). Subsequent post-hoc one-way ANOVAs for individual AST stages found significant CCI injury-dependent increases in set loss errors on the ED set-shifting ($F_{4,\ 34} = 4.517$; $p < 0.005$) and R3 stages ($F_{4,\ 34} = 3.725$; $p < 0.05$), as well as a trend for higher set loss errors on the second reversal (R2; $F_{4,\ 34} = 2.316$; $p = 0.078$). Post-hoc analyses also revealed that, on the ED stage, rats in the TBI (2.8 mm) group performed significantly worse than the naïve rats ($p < 0.05$), whereas the TBI (3.0 mm) group displayed significantly higher set loss error occurrences, compared to both the naïve and sham groups ($p < 0.05$; Table 2). Similarly, post-hoc analyses for individual group comparisons on the third reversal stage revealed a significant injury effect for the TBI (2.8 mm) group, relative to both the naïve and the TBI (2.6 mm) groups, observed as increased set loss errors, therefore a cognitive deficit representing the loss of contingency rule acquisition in this group ($p < 0.05$; Table 2). No other main effects or interactions were revealed on any other test stages.

Histology: Cortical lesion volume

At 5 weeks postsurgery, CCI injury induced impact depth-dependent increases in mean cortical lesion volume (Fig. 6). Mean cortical lesion volume for the TBI (3.0 mm) cortical deformation depth group resulted in the largest cortical injury ($59.3 \pm 5.4\ mm^3$), followed by the TBI (2.8 mm) deformation depth group ($49.9 \pm 3.3\ mm^3$) and the TBI (2.6 mm) deformation depth group ($45.1 \pm 3.0\ mm^3$). There was a statistically significant difference in cortical lesion volume assessments between the TBI (3.0 mm) and the TBI (2.6 mm) deformation depth groups ($t_8 = -2.29$; $p = 0.05$). No other group comparisons were significant (Fig. 6).

Discussion

This study set to investigate putative impairments in frontal cortex-mediated executive function and behavioral flexibility in a

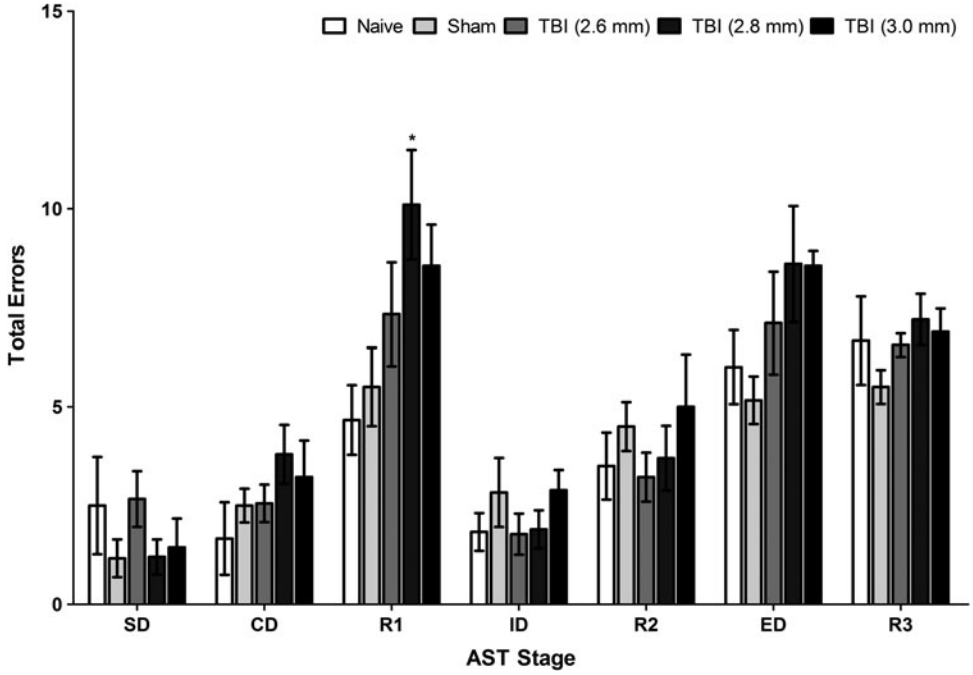

FIG. 5. Effect of controlled cortical impact (CCI) injury (2.6, 2.8, and 3.0 mm cortical deformation depth) on total response error rates in the AST. CCI produced a significant overall injury group effect ($p = 0.05$), as well as significant individual stage group effect on R1, with TBI rats displaying higher rates of total response errors while learning the contingency rule. Specifically, the TBI (2.8 mm) rats displayed significantly higher total error rates on R1, compared to naïve rats ($^*p < 0.05$, Newman-Keuls' post-hoc tests). Data are expressed as mean trials to criterion ± standard error of the mean ($n = 6$–9/group). TBI, traumatic brain injury; SD, simple discriminations; CD, compound discrimination; ID, intradimensional; ED, extradimensional; AST, attentional set-shifting test.

rodent model of TBI by subjecting rats to a CCI of varying cortical deformation depths (2.6, 2.8, or 3.0 mm) and assessing impact depth-dependent cognitive performance deficits in attentional performance on the AST. We found that TBI produced cortical impact depth-dependent impairments in ED set-shifting and stimulus reversals, manifested as a significant increase in the number of trials to reach criterion of six consecutive correct responses, as well as increased total response errors and set loss errors (i.e., failure to maintain acquisition of correct stimulus contingency), when assessed at 4 weeks postinjury. Specifically, rats subjected to the least injurious TBI condition (2.6 mm impact depth) did not display significant cognitive deficits in the AST; however, rats in the TBI (2.8 mm) group showed impaired cognitive performance throughout all the measures evaluated in this task, such as trials to reach criterion, total errors, and set-loss errors, whereas rats in the TBI (3.0 mm) group also exhibited selective ED set-shifting deficits in trials to criterion and set-loss errors. Moreover, as a validation of surgery accuracy and cortical impact depth-dependent injury severity, the TBI (3.0 mm) group displayed a significant increase in return to righting ability, as well as significantly larger cortical lesion volumes, in comparison to the TBI (2.6 mm) group. Conversely, there were no differences between sham (i.e., rats that received craniectomies) and naïve (i.e., no manipulation) animals, suggesting that this task is not differentially impacted by anesthesia and/or craniectomy. Taken together, these results provide a novel assessment of complex, frontal cortex-mediated cognitive processes, namely, executive function and behavioral flexibility, which were sensitively and reliably disrupted in our model of TBI. These findings are of significant relevance for expanding the validity of experimental models of TBI, by mimicking and characterizing higher function cognitive sequelae in humans, with the overarching goal of improving rehabilitation and pharmacological treatment paradigms in the clinical setting.

ED set-shifting, as a measure of cognitive flexibility, involves using environmental feedback to inhibit attending to a previously valid set or rules and begin to implement a set of rules involving a new pattern of reinforcement.[28,30] It has been reported that lesions

Table 2. Mean Set Loss Errors[a]

Group	*SD*	*CD*	*R1*	*ID*	*R2*	*ED*	*R3*
Naïve	0.00	0.33	0.83	0.00	0.00	0.00	0.33
Sham	0.17	0.33	0.67	0.33	0.33	0.33	0.33
TBI (2.6 mm)	0.44	0.67	0.22	0.11	0.11	0.67	0.44
TBI (2.8 mm)	0.22	0.67	0.89	0.11	0.22	0.78*	1.22*,†
TBI (3.0 mm)	0.22	0.89	0.56	0.22	0.67	1.11*	1.00

The repeated-measures ANOVA revealed a significant injury effect of group ($p < 0.001$), with Newman-Keuls post-hoc tests determining that the TBI (2.8 mm) and TBI (3.0 mm) deformation depth groups displayed significantly higher set loss errors throughout the AST stages, compared to controls. Specifically, the TBI (2.8 mm) group performed significantly worse in the ED stage than naïve rats ($^*p < 0.05$), whereas the TBI (3.0 mm) group displayed significantly higher set loss error occurrences, compared to both the naïve and sham groups ($^*p < 0.05$). Rats in the TBI (2.8 mm) group also displayed acquisition loss of contingency rule at higher rates, relative to both the naïve and the TBI (2.6 mm) groups, on the R3 stage ($^†p < 0.05$; $n = 6$–9/group).

[a]Occurring after 50% or more of the contingency rule has been acquired within a stage, therefore after three, four, or five consecutive correct responses were recorded, without actually reaching criterion of six consecutive correct responses.

SD, simple discriminations; CD, compound discrimination; ID, intradimensional; ED, extradimensional; ANOVA, analysis of variance; TBI, traumatic brain injury; AST, attentional set-shifting test.

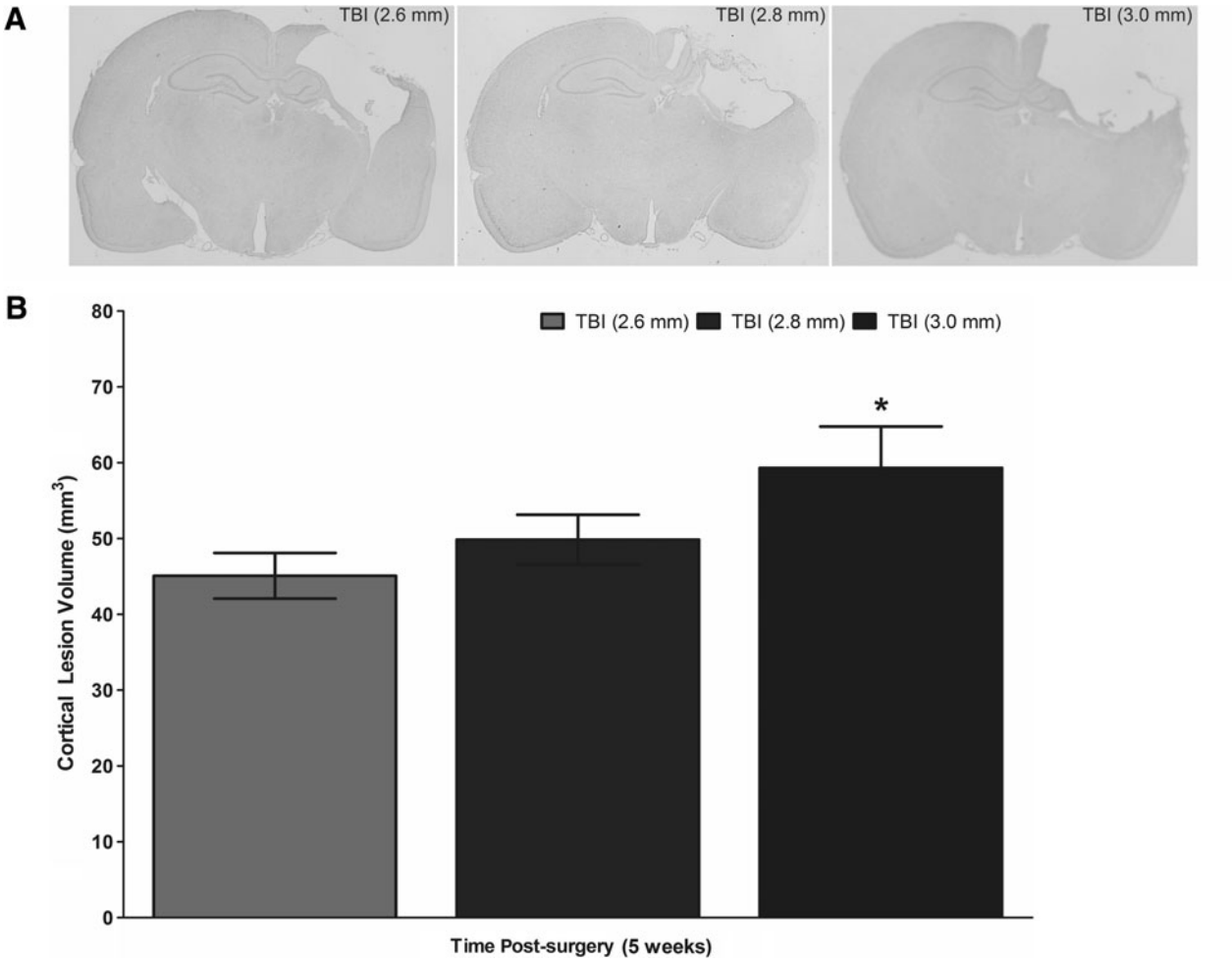

FIG. 6. (**A**) Panels are representative depictions of the lesion within each group at the level of the dorsal hippocampus. (**B**) Mean (±standard error of the mean) cortical lesion volume (mm^3) 5 weeks after controlled cortical impact (CCI) injury at three levels of injury (2.6, 2.8, and 3.0 mm cortical deformation depth, left to right, respectively). CCI injury produced a significantly larger cortical lesion volume in the TBI (3.0 mm) group, in comparison to the TBI (2.6 mm), deformation depth group (*$p = 0.05$). TBI, traumatic brain injury.

in the primate dorsolateral PFC and the analogous rodent medial PFC (mPFC) selectively alter ED set-shifting performance,[28,49] thus providing the basis for an expanding body of scientific literature examining executive function in a variety of experimental models of primarily psychopathological conditions affecting the PFC. The WCST is a multi-factorial test that requires the functional integrity of a widespread neural network of anatomical regions,[26] such as the PFC, parietal cortex, basal ganglia, and hippocampus,[50] all of which are brain regions vulnerable to damage, regardless of the profile of injury, and play a direct role in observed neurobehavioral sequelae.[51–53] This neuropsychological test of executive function has been validated across a myriad of pathological states involving PFC dysfunction, including brain trauma.[21,23,25,26] Moreover, a growing body of literature indicates that executive dysfunction is directly related to the level of brain injury severity in TBI patients,[23,54,55] with a significantly positive correlation also existing between injury severity and the percentage of perseverative and random errors.[24] Similarly, in the present study, CCI injuries at a 2.8- and 3.0-mm cortical impact depth (moderate-to-severe levels), but not a 2.6-mm cortical depth (milder), induced cognitive impairments in ED set-shifting and stimulus reversals of the AST, a rodent task designed to engage similar brain constructs as the WCST in humans or primates. Moreover, performance impairments on the WCST in various TBI population samples have been associated with increased supervision needs in severe TBI patients,[22] younger age in children at time of injury,[56] as well as lower circulation of hemoglobin,[57] and lower regional brain metabolism[58] in the right PFC. Of note, neuropsychological recovery after TBI occurs in a variable time-dependent manner across individuals and may continue for several years after injury.[59]

Neurobehavioral deficits, other than executive dysfunction, have been previously reported as dose dependent of injury severity after CCI injury.[18,60] Differential effects of injury severity were reported in rats after mild (20 pounds per square inch [psi] impact velocity, 1.5 mm depth of tissue compression) versus moderate CCI (30 psi, 2.0 mm), with moderate CCI producing greater impairment of motor function in a forelimb test and greater cortical tissue and hippocampal cell loss than mild CCI, albeit no CCI severity-dependent effects were detected on working memory, measured in the radial maze spontaneous alternation task.[60] In C57BL/6 mice,

CCI of varying impact speeds (5.0–7.5 m/sec) resulted in differences across injury severity levels in the standard and reversal MWM and novel object recognition tests, as well as, to a lesser extent, in the passive avoidance and tail suspension tests, whereas sensorimotor and learning impairments also displayed direct correlations with cortical lesion volumes and neuronal cell loss in subregions of the hippocampus.[18] In our study, there was no direct correlation between attentional performance and cortical lesion volumes; however, such analysis may be challenging, given the complexity of the AST design, with multiple stages assessing various facets of cognitive functioning. Nevertheless, our findings showing TBI-induced deficits in attentional performance at more severe levels of injury severity (2.8 and 3.0 mm impact depth), but not at milder levels (2.6 mm), are in accord with clinical studies reporting no significant changes in WCST perseverative responses after mild TBI,[61] as well as no measurable deficits in overall WCST performance in a subsequent study, where only patients with moderate-to-severe TBI showed larger deficits on several WCST indices at approximately 2.5–3.5 years postinjury.[23] The time interval between head trauma and EF assessment in these studies is comparable to the one implemented in the current study (i.e., 4 weeks postinjury), considering that, during adulthood, each rat month is approximately equivalent to 2.5 human years.[62,63]

All rats in the control groups successfully completed training and testing procedures, whereas in groups subjected to injury, 2 rats were eliminated because they did not complete training and 3 more did not complete the behavioral test procedure (1 in the TBI [2.6 mm] group, 2 in the TBI [2.8 mm] group, and 2 more in the TBI [3.0 mm] group). Though this attrition rate, even at its highest (18.18%), does not surpass previously published drop-out percentages in the AST,[20,38] it may create a selection bias that could ultimately lead to an underestimation of injury effects on cognitive performance, particularly for the TBI (3.0 mm) group. Data revealed that injury-related impairments in behavioral flexibility were slightly more robust in the TBI (2.8 mm) group than the TBI (3.0 mm) group, albeit the TBI (3.0 mm) group displayed, as anticipated, the largest cortical lesion volume group average, with a possible explanation for this behavioral paradox involving interhemispheric interactions under high processing demands. Specifically, cross-hemispheric compensatory recruitment by colossal interactions[64,65] may suffer from an interference effect through partially functional brain regions ipsilateral to injury, such as the hippocampus, in the TBI (2.8 mm) group, as opposed to reduced hemispheric inhibition upon more extensive hippocampal damage in the TBI (3.0 mm) group, hence leading to augmented behavioral deficits in the former group postinjury.

A common sequela after head injury is olfactory dysfunction (i.e., impaired sense of smell), either partial (i.e., hyposmia) or complete (i.e., anosmia), although only a small percentage of patients are aware of this disability and olfactory function appears to not recover in many TBI survivors.[66] Primary causes of post-TBI olfactory loss comprise distortion, overextension, and tearing of the olfactory nerves, as well as contusions or edema at the level of the olfactory bulbs and orbitofrontal (OFC) brain regions.[67,68] Specifically, a dose-response relationship between severity of brain injury and olfactory dysfunction has been reported in both pediatric[69] and adult[70] TBI patients. Concomitantly, the behavioral test used in this study involves learning contingency rules that incorporate stimuli across two perceptual dimensions (odor and medium), one of which is relevant for rule learning and the other serving as a distractor with its overlapping, nonreinforced, irrelevant cues. Using an olfaction-based paradigm could theoretically raise the question of whether injured rats display difficulties identifying specific odors necessary for rule learning, but our results suggest otherwise. There were no statistical differences in attentional performance throughout the AST stages in rats acquiring a cognitive set based on odor and switching to medium as the relevant dimension during the ED set-shift stage versus rats shifting stimulus dimensions in the opposite direction. Possible explanations for rats being able to easily discern various scents, even after significant brain injury, are that rodents have the ability for stereo odor localization,[71] they are macrosmatic (i.e., present a sensitive, well-developed olfactory system), with the olfactory bulb, anterior olfactory nucleus, and the primary olfactory cortex being important structures in the telencephalon,[72] and ablations of these regions or the olfactory-hippocampal pathway do not interfere with scent discrimination capabilities in rats.[73–75] In a broader sense, and considering that aromatic oil scents have been applied in discreet amounts on stimulus pots but above the human olfactory threshold, it is evident that any potential post-TBI olfactory dysfunction in rats may not be significant enough to affect AST performance. A recent study also supports this premise, because rats with bilateral frontal CCI injury showed decision-making deficits in a simple odor discrimination task (i.e., the dig task) using various scented sands to retrieve a food reward,[75] manifested as reduced cumulative proportions of correct scores, whereas parietally injured rats performed no different than shams. To an extent, the dig task is comparable with the SD stage of the AST, and parietally injured animals in our study did not display simple rule-learning deficits either. However, introducing more-complex contingencies, such as two overlapping stimulus dimensions, one of which is meant to act as a distractor, as well as shifting attention from one dimension to the previously irrelevant one, revealed distinct executive dysfunction in our TBI cohorts, with mono- or multi-synaptic PFC projections originating in the hippocampal formation likely being responsible for these behavioral impairments.[76,77] Future studies investigating deficits of executive function and behavioral flexibility in the AST after injury directly to the PFC are therefore warranted.

The neurobiological circuitry that facilitates attentional set-shifting and behavioral flexibility in rats is primarily mediated in the PFC and is contingent upon ascending projections from various neuromodulatory systems, such as norepinephrine (NE), but also dopamine (DA), serotonin (5-HT), and acetylcholine,[78–80] and manipulations of these modulatory neurotransmitters have differential effects on ED set-shifting performance, as well as reversal learning. For example, cortical NE depletion after 6-hydroxydopamine-induced lesions of the primary noradrenergic projections to the mPFC from the LC through the dorsal noradrenergic ascending bundle induced specific ED set-shift performance deficits.[33] Similarly, McGaughy and colleagues have reported that selective noradrenergic, but not cholinergic, deafferentiation to the rat mPFC through dopamine beta-hydroxylase/saporin infusions produced specific set-shifting impairments.[81] Moreover, enhancing noradrenergic tone by acute systemic administration of the α_2-adrenergic autoreceptor antagonist, atipamezole, improved ED performance, and this facilitation was blocked by local administration of the α_1-adrenergic receptor antagonist, benoxathian, directly into the mPFC.[30] ED set-shifting was also shown to be affected by DA manipulations in the PFC of marmosets,[82] as well as rats.[83] The potential influence of 5-HT on cognition is not as straightforward,[20] although 5-HT has been shown to facilitate glutamate transmission at pyramidal cell dendrites in the mPFC.[84] Depletion of 5-HT or destruction of cortical serotonergic projections produced prominent deficits in

reversal learning, which has been reported to be directly related to proper OFC function, rather than the mPFC.[29,85] Therefore, TBI-induced secondary sequelae involving disruption of endogenous neurotransmitter influences in the mPFC and OFC could be responsible for the impaired cognitive performance revealed in this study. This is consistent with a growing body of experimental TBI literature suggesting marked alterations in multiple neural pathways, such as neurotransmitter synthesis, release, or signaling, as well as effects on neurobehavioral outcome postinjury.[41,48,86–93]

To our knowledge, the current study comprises the first assessment of PFC-mediated executive function in a model of experimental TBI, and the results provide the impetus to integrate the AST in the standard neurotrauma behavioral battery after TBI in order to evaluate sensitive cognitive dysfunction and relevant therapies. Current studies in our laboratory are evaluating pharmacological and cognitive rehabilitation therapies alone and in combination as a relevant preclinical paradigm, as well as elucidating mechanisms underlying the neuropsychological deficits.

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Author Disclosure Statement

No competing financial interests exist.

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