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# Repeat Traumatic Brain Injury in the Juvenile Rat Is Associated with Increased Axonal Injury and Cognitive Impairments

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**Key Words**
Traumatic brain injury · Concussion · Repeat brain injury · Age · Axonal injury

**Abstract**
Among the enormous population of head-injured children and young adults are a growing subpopulation who experience repeat traumatic brain injury (RTBI). The most common cause of RTBI in this age group is sports-related concussions, and athletes who have experienced a head injury are at greater risk for subsequent TBI, with consequent long-term cognitive dysfunction. While several animal models have been proposed to study RTBI, they have been shown to either produce injuries too severe, were conducted in adults, involved craniotomy, or failed to show behavioral deficits. A closed head injury model for postnatal day 35 rats was established, and single and repeat TBI (1-day interval) were examined histologically for axonal injury and behaviorally by the novel object recognition (NOR) task. The results from the current study demonstrate that an experimental closed head injury in the rodent with low mortality rates and absence of gross pathology can produce measurable cognitive deficits in a juvenile age group. The introduction of a second injury 24 h after the first impact resulted in increased axonal injury, astrocytic reactivity and increased memory impairment in the NOR task. The histological evidence demonstrates the potential usefulness of this RTBI model for studying the impact and time course of RTBI as it relates to the pediatric and young adult population. This study marks the first critical step in experimentally addressing the consequences of concussions and the cumulative effects of RTBI in the developing brain.


## Introduction

Traumatic brain injury (TBI) remains the single most underaddressed pediatric health problem in the USA. Among the 1.6 million annual new head injury cases are a growing subpopulation of children and young adults who experience repeat TBI (RTBI). The most common cause of RTBI in this age group has been associated with sports and military activities. It is estimated that there are 300,000 sports-related TBI each year in the USA (Morbidity and Mortality Weekly Report, 1997). While the incidence of sports-related TBI varies with age [Kraus et al., 1990], it accounts for up to 40% of all brain injuries among children 5–14 years of age. Establishing the national incidence of RTBI in sports has been difficult, but smaller-scale studies reveal an estimated incidence of RTBI rang-

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ing from 5.6 to 34.9% [Collins et al., 1999; Guskiewicz et al., 2003; Jagger et al., 1984; Langburt et al., 2001; Müller and Neymann, 1967; Pellman et al., 2004; Slobounov et al., 2007]. While the number of annual cases of RTBI remains unclear, it is known that the risk for subsequent TBI increases with the number of previous concussions and with age [Annegers et al., 1980].

While a single mild concussive injury can produce numerous acute symptoms (including headache, dizziness, confusion, nausea, memory problems, fatigue, balance problems, attention and concentration deficits, and sleep disturbances), the magnitude and duration of these symptoms show a cumulative effect after RTBI [Guskiewicz et al., 2003]. Patients with multiple concussions have been shown to have a decreased rate of information processing [Gronwall and Wrightson, 1975], slower recovery of balance deficits [Slobounov et al., 2007], increased learning disabilities [Bijur et al., 1996; Collins et al., 1999; Gaetz et al., 2000; Wall et al., 2006], and increased difficulties in memory and concentration as well as headaches [Gaetz et al., 2000]. The magnitude and duration of these symptoms appears to be inversely related to age. High-school athletes have been shown to have longer memory dysfunction relative to age-matched controls than college athletes [Collins et al., 2006; Field et al., 2003]. The majority of these single and repeat concussive injuries produce symptoms in the absence of gross structural neuropathology. More recently, there is growing concern that an accumulation of mild concussive TBI in young adulthood results in early onset of cognitive and behavioral impairment [Guskiewicz et al., 2005], and some studies have demonstrated excessive accumulation of tau protein in the brains of ex-athletes who died of causes other than direct TBI [McKee et al., 2009; Omalu et al., 2006].

Since 1971, there have been 11 models of RTBI developed to address these clinical issues associated with RTBI. Although this list includes mouse, rodent, rabbit and pig models, many of these include open craniotomies, have significant mortality rates, are associated with significant pathology or present no behavioral deficits. Since the majority of concussions are (1) closed and (2) of a mild level of severity, (3) with low mortality rates that (4) exhibit symptoms in the absence of gross neuropathology, these same characteristics are required for any clinically relevant experimental model of RTBI. In our efforts to address concerns related to RTBI in children, our laboratory has developed a closed head, mild concussion model that generates measurable cognitive dysfunction in the juvenile brain.

## Materials and Methods

*Subjects*

Given that there is a high incidence of mild TBI and repeat concussions in school aged children and young adults, we proposed to use the postnatal day (PND) 35 rat for the following studies. While there are no exact guidelines for determining interspecies age equivalents, we have selected this age group based on several developmental profiles. PND 12–13 has been proposed to reflect a human newborn based on synapse formation and γ-aminobutyric acid synthesis [Romijn et al., 1991]. Sexual maturity is achieved at PND 60. Numerous metabolic developmental profiles including glucose metabolism [Nehlig et al., 1988] suggest that PND 35 rats reach approximately 90% of adult values. Based on this data, we believe that the PND 35 rat reflects a preadolescent human age group. Sprague-Dawley male rats used for acute histological changes were placed into the following groups: sham (n = 7), single TBI (n = 7) and RTBI 1d (2 injuries delivered 1 day apart; n = 13). PND 35 rats for the behavioral studies were placed into the following groups: sham (n = 12), single TBI (n = 12) and RTBI 1d (n = 12). The animals were maintained in standard vivarium housing with food and water ad libitum. All procedures were conducted with approval from the University of California at Los Angeles Chancellor's Animal Research Committee and were in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

*Injury Model*

Under isoflurane (2.0%/100% $O_2$) the head was shaven and the nose placed within a stereotaxic face mask to maintain anesthesia. The animals were placed on a heating pad with their head against a shaped wooden block within the stereotaxic frame (fig. 1), without earbars. An electronically controlled pneumatic piston cylinder (Hydraulic Control Inc., Emeryville, Calif., USA) was mounted onto a stereotaxic micromanipulator (Kopf Instruments, Tujunga, Calif., USA) to allow for precise localization of the impact center. A mask was used to mark the center of impact (–3 AP, –4 ML relative to the bregma) and the injury tip was firmly zeroed against the skin. The piston was angled at 23° away from the vertical to allow the impactor to make contact perpendicularly to the head's surface. The impactor tip (5 mm diameter) displaced the head 8 mm at 36 psi. The head was free to move in the direction of the injury. This level of impact does not cause skull fractures. However, it does produce apnea and delays the toe pinch response and righting time.

*Immunohistochemistry*

The animals for acute histology were euthanized 24 h after the second injury and perfused with 4% paraformaldehyde and cryoprotected in 20% sucrose. Coronal sections (40 μm) were collected and washed in PBS before preincubation in normal serum block (0.1% bovine serum albumin/1.5% normal serum) for 1 h. The sections were incubated in the primary antibody overnight at 4°C (β-amyloid precursor protein, β-APP, 1:200, Zymed; glial fibrillary acidic protein, GFAP, 1:2,000, Chemicon). Endogenous peroxidase activity was blocked by incubation in 0.5% $H_2O_2$ for 30 min followed by 3 PBS washes. The sections were then incubated in the biotinylated secondary antibody (1:200), followed by avidin-biotin complex/horseradish peroxidase and developed using 0.05% diaminobenzidine/0.02% $H_2O_2$ as substrates for horse-

radish peroxidase. The sections were then mounted onto gelatin-coated slides, dried, dehydrated, coverslipped and analyzed microscopically. A separate set of coronal sections were mounted onto microscope slides and processed for Fluoro-Jade staining.

The number of positive β-APP blebs was counted in 0.5-$mm^2$ regions within the corpus callosum ipsilaterally and contralaterally in 3 coronal sections (–0.92, –3.14, –5.2 mm relative to the bregma). GFAP labeling was only qualitatively assessed, given the difficulty of counting individual reactive glial cells.

*Novel Object Recognition Task*

Given the more subtle nature of the histological damage, it was likely that traditional behavioral tasks used for TBI models may lack the sensitivity to detect deficits after a mild injury. Clinically, concussive injury results in memory disturbances in humans, and the novel object recognition (NOR) task is a well-characterized behavioral measure of hippocampally based working memory in the rat that has been validated in the juvenile age range [Reger et al., 2009]. It was for these reasons that the NOR task was used. The NOR testing chamber was a white plexiglass arena (juvenile: 52 × 52 × 9 cm) that maximized exploratory behavior while minimizing incidental contact with testing objects. The dimensions of this apparatus were previously determined to be most appropriate for the PND 35 rats [Reger et al., 2009]. The room lighting was kept at a dim, ambient level. The rats were always transported from the home cage in the testing room to the testing chamber in a plastic standard vivarium cage covered by a white towel. The objects used for NOR testing were made of easy-to-clean materials such as rubber, plastic, glazed ceramic and glass, and consisted of a variety of household items and pet toys. The object sizes were age appropriate [Reger et al., 2009], i.e. no taller than twice the size of the rat, and it was important that objects did not resemble living stimuli (i.e. no eye spots or animal shapes). The arena and all objects were cleaned with 70% ethanol to remove odors between rats. The objects were secured to the testing chamber floor by Velcro and positioned centrally near the back wall of the chamber. Black permanent marker was used to shade from the tip of the rat's nose to between the ears, which allowed the tracking system (SMART; San Diego Instruments) to measure the time spent interacting with the objects. Digitally, a circular zone was created around each object so that movement ≤5 cm from the object's center could be detected as object interaction by the tracking system.

The NOR procedure was based upon the original Ennaceur and Delacour [1988] procedure. It consisted of a habituation phase followed by a testing phase. The habituation phase allows the animal to become acclimated to the new environment and allows assessment of the animal in the environment before and after injury, termed the 'open field' test. During the habituation phase, each rat was allowed 10 min to freely explore the empty arena 3 days prior to injury. During the open field testing, the animals were given 5 min to explore 1 day before and after the last injury. The duration of time and the distance spent within 3 zones (periphery, middle and inner zones) was determined for both days to determine anxiety effects of this injury.

The testing phase consisted of (1) a familiarization trial followed by (2) a test trial. During the familiarization trial, a rat was placed in the arena containing 2 identical objects, and given 5 min to interact and explore. The test trial was administered 1 or 24 h after the familiarization trial, and the animals were placed in the arena and allowed to explore for 5 min with 1 familiar object and 1 novel object. The duration of time spent with each object and the number of encounters were determined by the SMART tracking system.

**Fig. 1.** RTBI model. Anesthetized animal is placed against a wooden L-shaped frame secured within a student stereotaxic frame.

*Statistics*

All data are expressed as means ± SEM. To evaluate within-group performance, the percentage of total object interaction time (in seconds) spent with the novel object was compared to 50%, utilizing one-sample t tests. ANOVA was utilized for between-group analyses of memory performance. When appropriate, significant differences between levels of a factor were detected post hoc with a Bonferroni-adjusted $\alpha \leq 0.05$.

## Results

*Physiological Changes after Injury*

The physiological changes after injury are compared in table 1. There were no significant differences in the starting weights between groups. Following closed head injury, the animals demonstrated a period of apnea followed by a prolonged period of unresponsiveness to toe pinch reflex and righting delay. A single impact resulted in average apnea of 4.1 ± 1.1 s. There were no differenc-

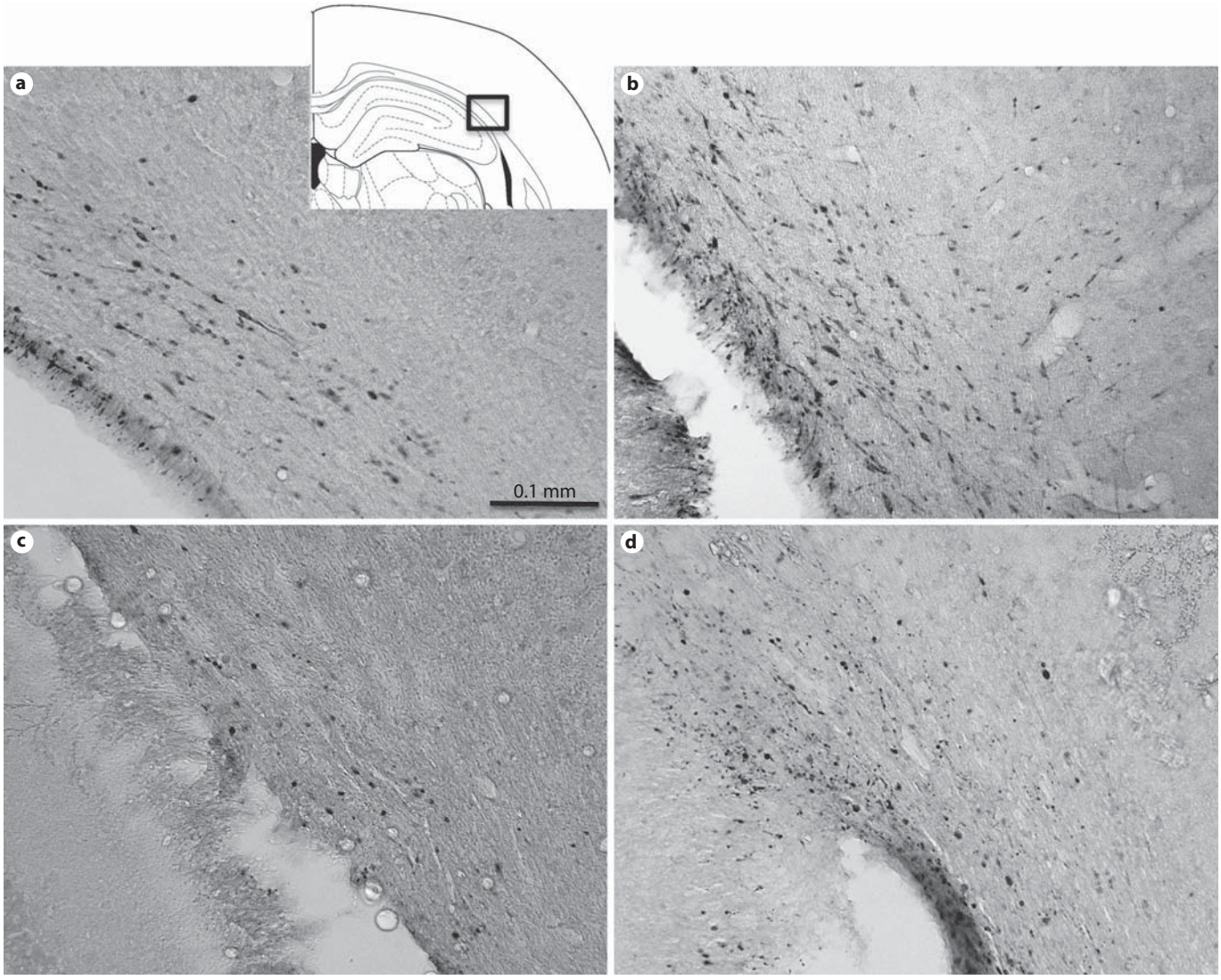


**Fig. 2.** β-APP immunohistochemistry of the grey-white matter junction under the impact site in single TBI (**a**, **c**) and RTBI (**b**, **d**) animals at 1 day after the last injury. Inset: region from which the photos were taken.

**Table 1.** Physiological changes after RTBI

| Groups | Weight g | Apnea (1st), s | Apnea (2nd), s | Toe pinch (1st), s | Toe pinch (2nd), s | Righting (1st), s | Righting (2nd), s | Mortality, % |
|---|---|---|---|---|---|---|---|---|
| Sham | 137 ± 3.4 | x | x | x | x | 21 ± 5.6 | x | x |
| Single injury | 147 ± 3.3 | 4.1 ± 1.1 | x | 50 ± 2 | x | 275 ± 21 | x | x |
| RTBI 1d | 138 ± 2.9 | 6.4 ± 1.3 | 8.2 ± 1.4 | 72 ± 12 | 68 ± 6 | 291 ± 24 | 291 ± 32 | 10 |

Values denote means ± SEM. $p < 0.05$ relative to first injury.

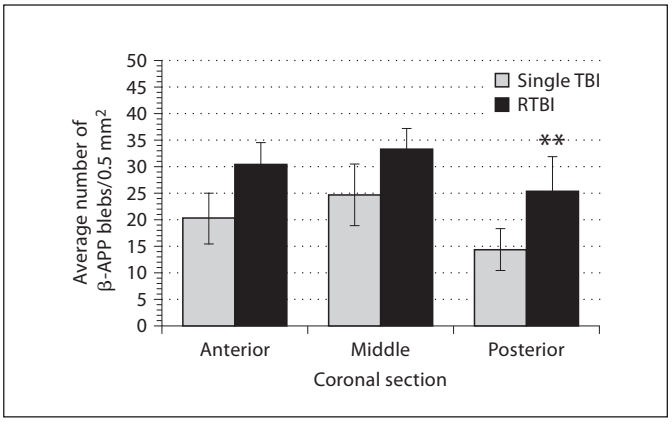


**Fig. 3.** Average number of β-APP-positive blebs from 3 regions of the grey-white matter junction in 3 coronal sections of single TBI and RTBI animals. RTBI animals showed a significantly greater number of β-APP-positive immunolabeling than single-impact animals. ** $p < 0.01$.

es between first-impact apneas between groups or relative to second-impact apnea. A single impact produced average toe pinch and righting times of 50 ± 2 and 275 ± 21 s, respectively. The duration of toe pinch response and righting did not differ between the first impact between groups or when compared to the second impact. The RTBI groups showed a 10% mortality rate, while the sham and single-impact groups had no deaths.

*Histology*

While fluid percussion, weight drop and controlled cortical impact models of injury in rodents can induce robust measurable cell loss, this closed head injury model at this level of injury severity does not. Fluoro-Jade staining for single and repeat TBI was negative in the cortex and hippocampus. Axonal damage was assessed by β-APP immunohistochemistry and astrocytic response via GFAP labeling. Brains from sham animals, and within the hemisphere contralateral to the side of impact in injured animals, did not show positive β-APP immunolabeling. Ipsilateral white matter tracks under the impact site showed positive β-APP immunolabeling in all 3 coronal sections for RTBI animals relative to single-injured animals 1 day after the last impact (fig. 2, 3). While anterior and middle coronal sections showed this trend, only the difference in the number of β-APP cells between single and repeat TBI in the posterior section achieved significance.

GFAP immunohistochemistry showed normal labeling of processes in the sham hippocampus and cortex (fig. 4a). One day following single impact, GFAP labeling in the grey-white matter junction under the impact site did not appear to be more intense than in the sham animals (fig. 4b). One day after an RTBI, there was a bilateral increase in GFAP labeling with clear morphological changes in the astrocytes around the site of impact (fig. 4c, d). This bilateral increase in GFAP labeling was consistently observed among all RTBI animals.

*Open Field and Novel Object Tasks*

Following 3 days of habituation, all animals performed similarly in the preinjury open field. On the day of open field after the injury, all groups appeared to spend the majority of their time in, and had more entries into, zones 1 and 2, with fewer entries or time spent in zone 3 (fig. 5a, c). There were no statistical differences between the 3 groups in the time spent, the number of entries or the distance traveled in each of the zones ($F_{2, 15} = 0.003$).

Animals able to discriminate between the old and new objects should perform better than 50%. When the NOR testing was done 1 h after familiarization, all 3 groups discriminated between the new and old objects (fig. 6b). The percent time spent with the novel object was 67.8 ± 7.2, 63.4 ± 5.5 and 68.3 ± 7.1 s for the sham, single-injured and RTBI groups, respectively. The percent time spent with the novel object after a 1-hour delay was not significantly different between the groups ($F_{2, 14} = 0.164$). When the animals were challenged with a 24-hour interval, both the single-impact and RTBI groups showed a significant decrease in the percent time spent with the novel object relative to the sham animals ($F_{2, 14} = 10.45$; $p < 0.002$) (fig. 6c).

## Discussion

In the past, concussions or mild TBI have been dismissed as insignificant injuries since most individuals appeared to recover with time. However, as several scientists have stated, mild TBI are only 'mild' when they happen to someone else. Using advanced methods such as diffusion tensor imaging (DTI), individuals with concussions demonstrate white matter abnormalities that correspond to physical, emotional and cognitive symptoms [Wilde et al., 2008; Wozniak et al., 2007] and/or electrophysiological measures such as magnetoencephalography [Huang et al., 2009]. Complicating these postconcussive symptoms are the issues of subsequent concussions and their effect on the duration and magnitude of symptoms, especially in the pediatric population, where RTBI

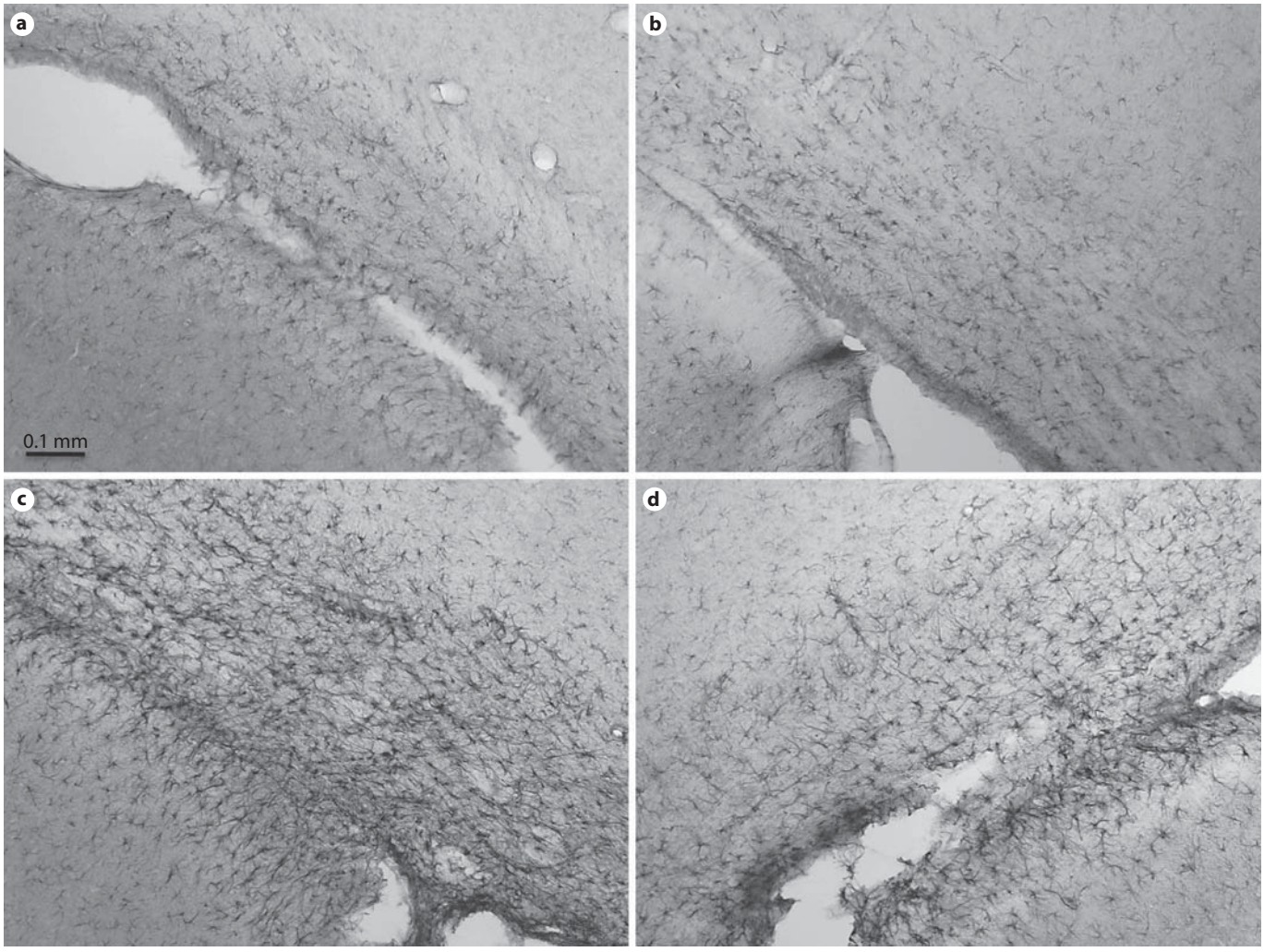


**Fig. 4.** GFAP-positive labeling in the grey-white matter junction at the site of impact in ipsilateral sham (**a**), single impact (**b**), ipsilateral RTBI (**c**) and contralateral RTBI (**d**).

is a serious risk. The results from the current study demonstrate that an experimental closed head injury in the rodent with low mortality rates and absence of gross pathology can produce measurable cognitive deficits in a juvenile age group. The introduction of a second injury 24 h after the first impact resulted in increased axonal injury, astrocytic reactivity and increased memory impairment in the NOR task.

*Structural Changes in White Matter*

The pathology associated with concussions is by nature subtle and may not have been readily detectable using standard methods of clinical imaging in humans, or experimental histological approaches in animals have not been sensitive enough to detect mild changes. While axonal injury has been detected after experimental brain injuries using β-APP and neurofilament immunohistochemistry [Povlishock and Pettus, 1996], DTI has been instrumental in illuminating white matter damage after human mild TBI. DTI has demonstrated alterations in fractions in regions of the corpus callosum and frontal white matter following mild TBI in adults [Bazarian et al., 2007; Kraus et al., 2007; Lipton et al., 2008] and pediatric patients [Wilde et al., 2008; Wozniak et al., 2007]. Decreased white matter fractional anisotropy has been found to be associated with slower motor speed, executive function deficits and severity of postconcussive symptoms in both children and adolescents [Copeland et al., 2008; Niogi et al., 2008; Wilde et al., 2008; Wozniak et al., 2007]. This more subtle evidence of white matter

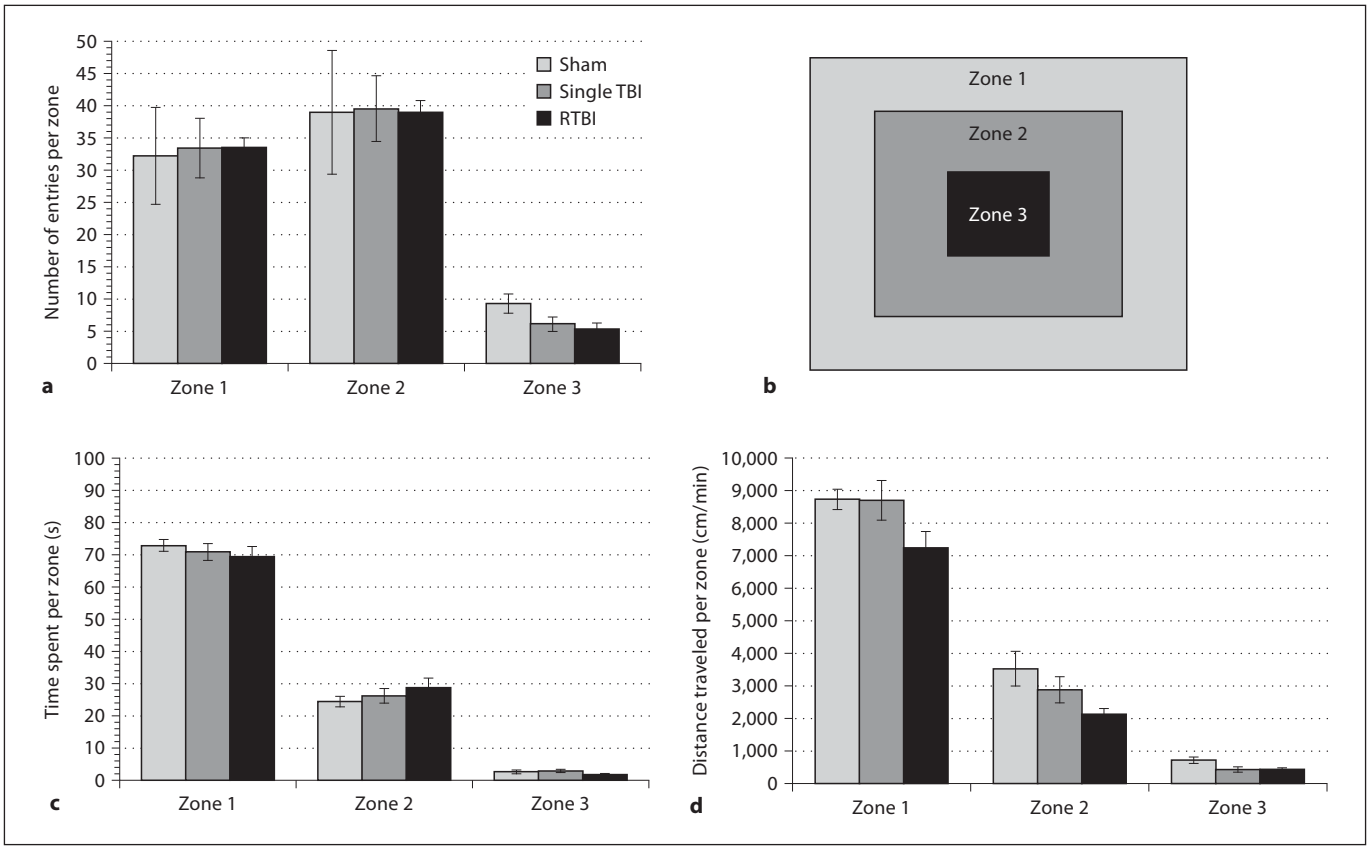


**Fig. 5.** Average number of entries (**a**), time spent (**c**) and distance traveled per zone (**d**) during the open field test 1 day after the last injury. Columns: means. Whiskers: SEM. **b** Diagram of the zones.

injury was also observed 24 h after a single impact in the current study, and increases with a second insult endured 24 h later. We are unaware of any publications utilizing DTI to assess axonal injury following multiple concussions or in animal models of RTBI. The causal relationship between observed axonal injury and functional consequences has yet to be experimentally demonstrated.

In addition to white matter damage, TBI is often associated with increased reactive astrocytosis [Baldwin and Scheff, 1996; Regner et al., 2001; van Landeghem et al., 2006]. While there was a slight increase in GFAP labeling 1 day after a single injury, the further increase with RTBI in the present study has also been observed in adult rodents with repeat weight drop [Hamberger et al., 2009] and PND 11 rats with 2 or 3 closed head impacts [Huh et al., 2007]. The bilateral nature of the elevated GFAP labeling and increased reactive profile of the astrocytes at the grey-white matter junction reveals the diffuse nature of the injury model used in the current study.

*Functional Changes in Cognition and Behavior*

Concussions have been shown to cause slowed mental processing, decreased concentration, inability to focus, impaired memory and learning deficits [McClincy et al., 2006; Sim et al., 2008]. Cognitive outcomes are exacerbated with RTBI in athletes of various ages [Collins et al., 2002; Gronwall and Wrightson, 1975; Moser et al., 2005]. While many of these cognitive issues may be considered 'mild' and may not interfere with normal daily activities, when TBI patients are mentally challenged, deficits can become apparent. As the task difficulty or number of tasks increases, mild TBI groups show more severe deficits in performance [Cicerone, 1996; Mathias et al., 2004]. Consistent with these clinical reports, the single and repeat TBI groups in the present study were able to differentiate between the old and new objects when tested after a 1-hour interval. But when they were challenged to discriminate the objects after the more difficult 24-hour interval, the RTBI group was significantly impaired in both distance traveled and time spent with the new object.

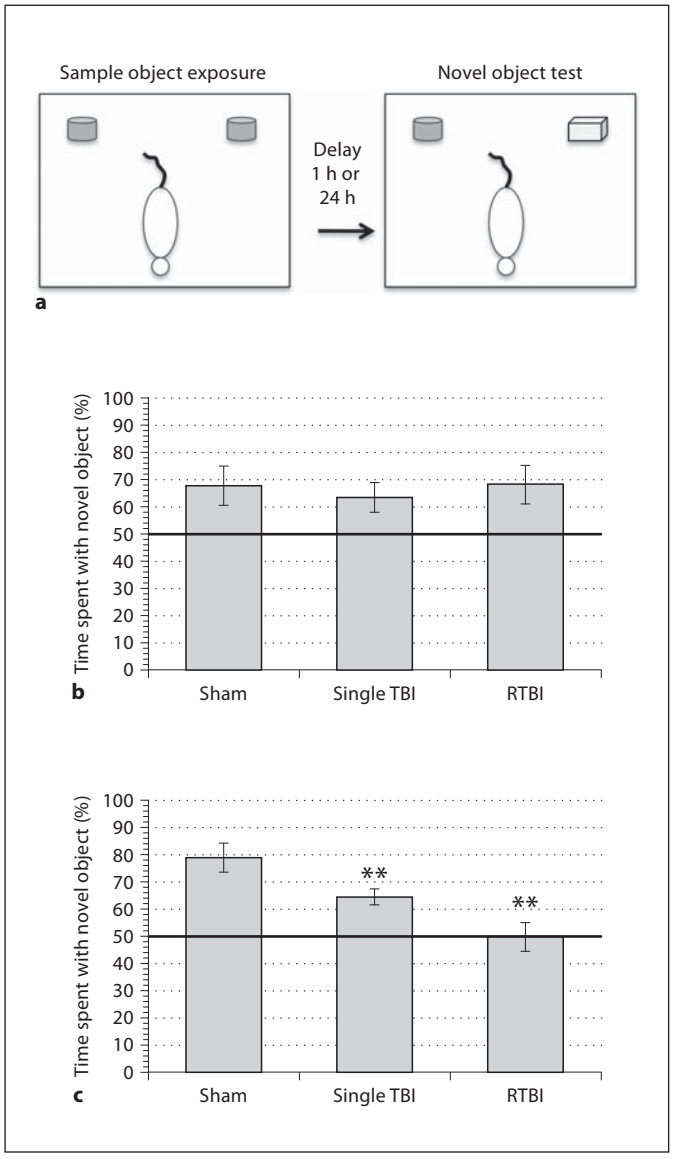


**Fig. 6. a** Diagram of the novel object recognition task. **b**, **c** Percent time spent with novel object with intertrial period of 1 h (**b**) and 24 h (**c**) at day 2 after injury. ** $p < 0.05$ relative to sham animals.

*Limitations and Future Directions*

It was our intention to examine the acute effects of repeat mild head injury in a relevantly aged animal model. However, there are limitations to both the histological and behavioral outcomes of this current study. Typically, injury-induced pathology can continue to develop for weeks after TBI. Cell loss assessed by cell-type stereological counting or markers of cell death are usually conducted 7–14 days after injury. Markers of axonal injury are usually assessed 72 h after injury. The current study does not address the future development of the pathology, and a time course of these outcome measures will be analyzed in the future to fully characterize the injury development. In addition, while the use of the NOR task after TBI is not as traditional as the use of the Morris water maze, it has been previously used [Biegon et al., 2004; Wakade et al., 2010]. Future studies will have to compare the sensitivities of the NOR task and Morris water maze cognitive measures following mild injuries.

In addition to histological and behavioral issues, mortality in any RTBI model should be addressed. Human single-impact mild TBI does not have to entail loss of consciousness, and has no mortality. A single impact in the current study had no mortality. Mortality from mild RTBI is rare and has only rarely been observed in association with second-impact syndrome [McCrory, 2001]. The 10% mortality reported in this study may suggest that the injury was more severe than a mild injury, but it was only observed among RTBI animals when injuries were 24 h apart. Other injury intervals (3 or 5 days) show 0% mortality (data not presented), suggesting that there is something additive about this specific interval.

The majority of head injuries occur in children, adolescents and young adults, especially with regard to sports-related concussions. While currently there are more than 20 expert guidelines focused on managing adult athletic concussions, there are less than a handful of management guidelines or research studies focused on pediatric concussions or repeat concussions [Kirkwood et al., 2006]. It is clear from both our laboratory's previous work and clinical studies that children are not simply little adults, and that diagnosis, management and treatment options must be tailor made for different cerebral developmental stages. This study marks the first critical step in experimentally addressing the consequences of concussions and the cumulative effects of RTBI in the developing brain. Future studies regarding the window of vulnerability, biomarkers of return to play, gender, effect of exertion, impact location and therapeutic options can now begin to be addressed experimentally.


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