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Juvenile traumatic brain injury evolves into a chronic brain disorder: behavioral and histological changes over 6 Months

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Abstract

Traumatic brain injury (TBI) refers to physical trauma to the brain that can lead to motor and cognitive dysfunctions. TBI is particularly serious in infants and young children, often leading to long-term functional impairments. Although clinical research is useful for quantifying and observing the effects of these injuries, few studies have empirically assessed the long-term effects of juvenile TBI (jTBI) on behavior and histology.

After a controlled cortical impact delivered to postnatal 17d rats, functional abilities were measured after 3, 5, and 6 months using open field (activity levels), zero maze (anxiety-like behaviors), rotarod (sensorimotor abilities, coordination, and balance), and water maze (spatial learning and memory, swim speed, turn bias). Sensorimotor function was impaired for up to 6 months in jTBI animals, which showed no improvement from repeated test exposure. Although spatial learning was not impaired, spatial memory deficits were observed in jTBI animals starting at 3 months after injury. Magnetic resonance imaging and histological data revealed that the effects of jTBI were evolving for up to 6 months post-injury, with reduced cortical thickness, decreased corpus callosum area and CA1 neuronal cell death in jTBI animals distant of the impact site.

These findings suggest that this model of jTBI produces long-term impairments comparable to those reported clinically. Although some deficits were stable over time, the variable nature of other deficits (e.g., memory) as well as changing properties of the lesion itself, suggest that the effects of a single jTBI produce a chronic brain disorder with long-term complications.

Keywords

traumatic brain injury; pediatric; behavior; neurodegeneration; MRI

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Introduction

Traumatic brain injury (TBI) has debilitating consequences and affects nearly 4,000 people in the United States each day (Morales, et al., 2005). By far, young men have the highest incidence of TBI. Historically, most research and clinical practice has focused on injuries in adults, as it has been assumed that the greater potential for neuroplasticity may partially ameliorate impairments in younger patients (Adelson, et al., 2013, Giza, 2006). However, those injured at a young age are more prone to long-term behavioral changes like attention deficit / hyperactivity, anxiety, depression, motor problems, and learning deficits (Ewing-Cobbs, et al., 1998, Geraldina, et al., 2003, Giza, 2006, Jorge, 2005, Jorge, et al., 2004, Levin, et al., 1982). Evidence suggests that juvenile TBI (jTBI) can result in increased severity of symptoms and worse outcomes compared with similar injuries in adult patients, perhaps due to disrupted neurodevelopment (Gerber and Coffman, 2007, Kramer, et al., 2008). Such symptoms and deficits may interfere with a patient's development, daily functioning, and quality of life, causing drastic alterations in their long-term health and well-being (Donders and Warschausky, 2007). Clinically, interest is growing in long-term analyses tracking juvenile injuries into adulthood (Barlow, et al., 2005, Beauchamp, et al., 2011, Beauchamp, et al., 2011).

As mentioned previously, characterization of the pathophysiological and functional changes following TBI have most often been reported in adult rodent models of controlled cortical impact (CCI) (Adelson, et al., 2013). More recently, work with juvenile models of TBI has been gaining momentum, using protocols modified from adult models (Adelson, et al., 2013). To mimic the contusion and parenchymal injury following TBI, a common juvenile injury model uses young rats aged 17 days old (P17) and CCI (Adelson, et al., 2013). In a comparative study of the functional (water maze) and histological changes after CCI with injuries of 0.5, 1.0, and 1.5 mm depth on either P7, P17, or adult rats (Adelson, et al., 2013), it was suggested that histopathological changes persist at both early and delayed timepoints up to 28 days post-injury. Functional deficits after adult TBI in rodents include hyperactivity, depression- and anxiety-like behaviors, sensorimotor problems, and learning and memory difficulties for up to 20 days post-injury, with some deficits, including affective changes, lasting up to 6 months (Jones, et al., 2008). In jTBI models, affective changes, as well as sensorimotor deficits, have been observed up to 2 months post-injury (Ajao, et al., 2012, Hartman, 2011). However, differences involving anxiety-like behaviors fluctuated up to the study terminus (Ajao, et al., 2012), suggesting that behavioral and cognitive changes may continue past the 6-month period. In that study, we also published data showing persistent motor deficits at 2 months after a cortical impact in juvenile rats (Ajao, et al., 2012), and memory problems have been reported up to 3 months in a similar juvenile rat injury model (post-natal day 17) (Adelson, et al., 2000). Long-term physiological findings from animal jTBI models have suggested reduced brain plasticity, increased dopamine production, and white-matter dysfunctions compared to non-TBI animals (Ajao, et al., 2012, Prins and Hovda, 2003, Walter, et al., 2004). Together, these data indicate that experimental TBI models can exhibit complex behavioral changes similar to clinical observations in humans following an early life injury.

We previously studied sensorimotor function, spatial memory and anxiety-like behaviors at 1 and 2 months after a controlled cortical impact (CCI) injury to 17 day old (juvenile) rats (Ajao, et al., 2012). An analysis of water maze strategy use highlighted less reliance on "spatial" strategy use by jTBI rats, which emerged 2 months after injury (Pop, et al., 2013). As indicated by Dr Adelson and collaborators, most of the studies using CCI models have focused on potential new drugs for immature brain, but little was done to define the functional as well as tissue changes in jTBI (Adelson, et al., 2013). Altogether, our recent works suggest that the behavioral profile of injured animals is still evolving at least 2

months after injury and may include additional behavioral domains. Therefore, we further examined the effects of a cortical impact to the juvenile rat behavior repertoire at 3, 5 and 6 months post-injury and tissue properties at 3 and 6 months post-TBI. Specifically, we assessed changes in general activity (open field), anxiety-like behaviors (zero maze), sensorimotor, coordination and balance abilities (rotarod, foot fault, balance beam), and cued and spatial learning / memory (water maze) to provide a complete and relevant description of the long-term sequelae following jTBI in rodents. In parallel, we evaluated characteristics of the lesion site and surrounding brain structures using magnetic resonance imaging (MRI) and histological preparations including neuronal and glial markers.

Material and Methods

Animals

All protocols and procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Juvenile male Sprague-Dawley rats (17 days old, Harlan, Indianapolis, IN) were housed with their dams on a 12-hour light-dark cycle at constant temperature and humidity. Pups were weaned at 24 days of age (7 days after surgery), housed 2 rats per cage, and fed with standard lab chow and water *ad libitum*. Two groups of animals, sham (n=7) and jTBI (n=8), were studied longitudinally for all behavioral tests at 3, 5 and 6 months post injury. This same group was used for MRI at 3 and 6 months and histological analyses at 6 months.

Juvenile traumatic brain injury model

The rats were given a CCI or sham surgery as described previously (Ajao, et al., 2012). Briefly, all animals were anesthetized using isoflurane at 2–3%, maintained at 0.5%, and placed into a stereotaxic frame (David Kopf Instruments, USA). A 5 mm diameter craniotomy opened the skull over the right fronto-parietal cortex (epicenter at –1 mm posterior and 2 mm lateral from *bregma*). The TBI group was given a CCI in the center of the craniotomy at a 20-degree angle using an electromagnetic impactor with a 3 mm diameter tip (Leica Microsystems Richmond, IL), at 6 m/s with an impact depth of 1.5 mm and duration of 200 ms. The sham group received the craniotomy but no cortical impact; the skin overlying the skull of both groups of animals was then sutured (Ajao, et al., 2012). Buprenorphine (0.01 mg/kg) was injected subcutaneously to reduce pain for all animals.

Behavioral Testing

Behavioral testing was administered to detect and track functional differences between sham and TBI animals 3, 5, and 6 months after injury. This long-term longitudinal design was chosen as a follow-up to differences observed at 1 and 2 months post-injury (Ajao, et al., 2012). To provide consistency, the same tests were administered in the same order at all three time points, by the same investigators (JK, DA) blinded to the experimental groups.

Foot Fault—Motor coordination and proprioception were assessed using the foot fault test, which consists of a rectangular wire mesh grid 2.5 cm × 30 cm. Rats were placed in the middle of the grid and their movements recorded for 60 seconds. Each paw slip through the grid was recorded as a fault. The sum of total faults at the end of the trial served as the dependent variable. Each animal was administered two trials spaced 30 minutes apart (Schmanke, et al., 1996).

Beam Balance—Motor coordination and activity levels were assessed using the beam balance task (Lekic, et al., 2011). This task consists of a plastic tube 60 cm in length and 2.5 cm in diameter, with demarcations every 2.5 cm. Animals were placed in the middle of the beam perpendicular to the length of the beam, and allowed to walk unimpeded for a 60

second trial. The distance covered during each trial was recorded as the dependent variable. Each animal was administered two trials of the beam balance test spaced 20 minutes apart.

Rotarod—Motor coordination and balance were further assessed using the rotarod test (Rotamex-5, Columbus Instruments, Columbus, OH; (Dunham, 1957 #1096; Jones, 1968 #1094). Each rat was placed on the rotarod, which consists of a rotating spindle 7 cm wide, and had to continuously walk forward to avoid falling. Each animal was given three blocks of two trials each (15 min apart), including 2 trials in which the rod rotated at 2 RPM and 2 trials in which the rod slowly accelerated from 2 RPM, increasing in speed by 2 RPM every 5 seconds. These were followed by a set of fast acceleration trials (initial 2 RPM speed with a 2 RPM increase every 3 seconds). An animal's latency to fall (in seconds) was used as the dependent variable; this variable is sensitive to both motor coordination and balance (Fujimoto, et al., 2004, Hamm, et al., 1994, Jones and Roberts, 1968).

Open field—General activity levels were assessed using the open field test (Hall and Ballechey, 1932). Plastic enclosures (50 × 36 × 45 cm) were used for this test, and each animal's behavior was monitored for 30 min with an overhead video camera using Noldus Ethovision software. The dependent variable for this test was distance traveled.

Zero maze—Anxiety-like behaviors were assessed using the elevated zero maze test (Granacher, 2003, Shepherd, et al., 1994), which consists of an elevated circular track 10 cm wide with a 100 cm outer diameter. Two sets of 30 cm high walls enclose two opposing quadrants of the track, leaving the other two opposing quadrants open and brightly lit. Each animal was observed for 5 min, and the dependent variable was the percentage of the trial spent in the enclosed or "dark" half of the maze.

Water maze—Spatial learning and memory were assessed using the water maze, which consists of tank, 100 cm in diameter, filled with opaque water. An 11 cm diameter escape platform was placed in the tank, and three days of testing were administered. Recording the animal with an overhead camera and the Noldus Ethovision system allowed for assessment of variables such as swim distance, swim speed, and relative angular velocity (left / right turn bias).

The first day of testing consisted of the cued learning paradigm, designed to test for non-associative factors, such as motivation, swimming ability, and vision that may complicate later spatial testing. For this task, the platform's surface was visible and raised 2 cm above the waterline, with a protruding 40 cm wooden rod attached to aid visibility. Animals were placed in the tank and required to simply swim to the visible platform to end the task. Each animal completed 10 trials consisting of 5 blocks of 2 trials each, with their starting location and that of the platform being shifted throughout the task. If the animal had not found the platform after 60 sec, it was guided to and placed on the platform, where it remained for 5 seconds. The dependent variable for the cued task was distance moved, defined as an animal's total swim distance for each trial.

Days 2 and 3 of the water maze consisted of the spatial paradigm, designed to measure an animal's ability to learn and remember the platform's location in the tank. During this paradigm, the platform's surface was hidden approximately 2 cm below the water's surface, and release points varied throughout each day although the platform remained stationary. As in the cued paradigm, animals were required to swim to the platform to end the task. Unlike the cued paradigm, hiding the platform's surface under the water's surface required animals to learn and navigate to the location using spatial cues. Each animal completed 10 trials consisting of 5 blocks of 2 trials each. If the animal had not found the platform after 60 sec, it was guided to and placed on the platform, where it remained for 5 seconds. The dependent

variable for the spatial task was cumulative distance to the platform, a variable sensitive to both distance and time defined as a cumulative total of an animal's distance from the platform as measured 5 times every second.

After each day of spatial testing, a probe trial was administered to measure an animal's ability to remember the platform's location. This trial was administered 1 hour after the final spatial trial of the day. Animals were allowed to search the tank for 60 sec. Dependent variables for this task included the number of times an animal entered the platform's previous location and percent time spent in the target quadrant.

Magnetic Resonance Imaging and Analysis

MRI was performed *in vivo* at 3 months followed by high resolution *ex vivo* imaging at 6 months as previously described (Ajao et al., 2012). Briefly, for *in vivo* MRI at 90 dpi, rats were anesthetized using isoflurane (1–2%), placed in a temperature controlled cradle and imaged on a 4.7T MRI (40 cm bore, Bruker Biospin, Billerica, MA). For *ex vivo* MRI at 180 dpi, animals were transcardially perfused with 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS), and the extracted brains were transferred to PBS before *ex vivo* scanning using the 11.7T MRI (8.9cm bore, Bruker Biospin, Billerica, MA). The use of two different field strengths did not alter the measurements performed in our experiments, as previously described (Ajao et al., 2012).

T2 weighted images (T2WI) from each MRI scan were imported to Cheshire image processing software (Hayden Image Processing Group, Waltham, MA) for volumetric analysis of 1 mm slices through the whole brain (*bregma* +5.0 to –9.0 mm), ipsilateral and contralateral hippocampus (*bregma* –1.8 to –6.8 mm), corpus callosum (CC) area in the hemisphere contralateral to the lesion (*bregma* –0.8mm) (Ajao, et al., 2012), and TBI lesion volume (mm³) on appropriate slices. Lesion was defined as regions containing abnormal hypo- and/or hyper-intense signals, where all tissue below the skull level was included. Hippocampal and corpus callosum measurements included all visible portions of these structures on selected coronal sections, which were chosen based on similarities of anatomical landmarks.

Thickness of ipsilateral and contralateral parietal cortex was obtained from MR images at 1 mm intervals for 8 coronal levels in total (*bregma* +2.8 to –4.8 mm), with the most anterior and the most posterior coronal slices having no visible lesion in any jTBI cases. For each slice, a line was drawn perpendicular to the direction of the perilesional parietal cortex, starting from the lateral edge of the corpus callosum and ending at the outer edge of the brain; these anatomical landmarks were kept consistent across slices of each animal for all MRI scans. To correct for individual brain differences, the injury and hippocampal volume data are represented as a % total brain volume and cortical thickness is shown as a ratio of % ipsilateral/contralateral values.

Tissue and Histological Analyses

At 6 months, rats were transcardially perfused with 4% paraformaldehyde and subsequently processed for immunohistochemistry as previously described (Ajao, et al., 2012). Following brain extraction, the CCI location (mm distance from anterior tip of brain to center of lesion cavity) was determined using laboratory calipers. Then, brains were sucrose fixed for 48 hrs, frozen on dry ice, and 50 µm coronal sections were cut at –20°C throughout the entire length of the brain on a cryostat (Leica CM1850, Leica Microsystems GmbH, Wetzlar, Germany).

Serial coronal sections spaced 2.4 mm apart were used for immunostaining and visualization with secondary antibodies for classical wavelengths (350, 488, or 594nm) or infra-red (700

or 800nm). Following washes in PBS, pH=7.4 (Fisher Scientific, Pittsburgh, PA), sections were blocked in 1% BSA (Sigma-Aldrich Co., St. Louis, MO) prior to overnight primary antibody incubation at 4°C in in PBS, pH=7.4, with 0.25% BSA and 0.25% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO). Determination of neuronal distribution was carried out using an antibody against neuronal nuclei (mouse anti-NeuN 1:1000 for classical and 1:500 for infra-red, Chemicon International, Temecula, CA), an antibody against myelin basic protein (mouse anti-MBP, 1:200 for infra-red, Chemicon International, Temecula, CA) and an antibody against neurofilament 200 (rabbit anti-N200, 1:200, Chemicon International, Temecula, CA). After rinsing in PBS, sections were incubated for 2 hours at room temperature with goat anti-mouse secondary antibody coupled with Alexa-Fluor-488 for classical staining or with Alexa-Fluor-800 for infra-red analysis (1:1000; Life Technologies: Invitrogen, Grand Island, NY), and subsequently washed in PBS prior to mounting all tissue on glass slides. Sections for infra-red analysis were first allowed to air-dry and then scanned using the same parameters for sham and jTBI at 21µm resolution (Li-Cor Odyssey Bio-Science, Lincoln, NE). Lastly, all stained sections were coverslipped with anti-fading medium Vectashield containing DAPI (Vector, Vector Laboratories, Burlingame, CA). Control sections, where primary or secondary antibodies were omitted, resulted in negative staining.

MBP analysis was performed as described previously (Ajao, et al., 2012). Briefly, an average immunoreactivity (MBP-IR) intensity value was obtained for each animal from identical region of interests (see supplemental figure 1) drawn on the contralateral CC using the Licor-Odyssey software for infra-red staining. For DAPI-positive nuclei in the CA1 region of the ipsilateral and contralateral hippocampus, coronal section at *bregma* level -3.4 mm were used for automated counting with Mercator software (Explora-Nova, La Rochelle, France).

Statistical analyses

All data are presented as mean $\pm$ SEM, with the exception of the water maze turn bias which uses 95% CI's. Statistical analyses and graphs were done using SPSS 17, Prism 6, and SigmaPlot. All behavioral data that contained a within-subjects component were computed using mixed-model, repeated-measures ANOVA to assess differences between groups over multiple trials. Each model contained three within-groups data points (3, 6, 9 months post-injury), as well as two levels of between-groups data (sham vs. TBI). The exception to this regarded the turn-bias, where one-sample t-tests were used to test whether each group exhibited a turn bias compared with baseline (no bias). MR and histological data with several coronal slices were also analyzed using a repeated-measures ANOVA to assess differences across the rostralcaudal axis of the brain. A one-way ANOVA with a Bonferroni correction was used for comparisons over time and group when more than two variable states were indicated (90 and 180 days, sham and jTBI). A student's t-test was used to assess differences between independent variables (e.g. one coronal level) between two groups (sham and jTBI).

Results

Neurological and behavioral testing

We did not observe any long-term functional differences in activity levels or anxiety-like behaviors as assessed with beam balance, open field, and zero maze at 3, 5, and 6 months post-injury. However, jTBI animals did exhibit persistent and emergent deficits in other domains.

Injured animals showed sensorimotor, coordination and/or balance deficits on the rotarod task compared to shams, $F(1, 13)=5.60$, $p<0.03$ (Fig 1A). These differences emerged for the faster acceleration rotarod paradigm, and represent a relatively consistent effect across time. The absence of any significant group by time point interaction suggests that these deficits are relatively persistent and stable; repeated exposure to this task did not significantly improve the performance of either jTBI animals or shams. Similarly, jTBI animals had more foot faults than sham animals, $F(1, 13)=8.70$, $p<0.02$ (Fig 1B). However, the presence of a group by time point interaction, $F(2, 26)=3.44$, $p<0.05$ (Fig 1B) indicates that jTBI animals improved by 6 months to the same level as that of sham animals, whose number of faults remained consistent over time (Fig.1B). In the water maze, jTBI animals showed a persistent left turn bias (Fig1C2), not exhibited by sham animals (Fig1C1), while swimming at 3, 5 and 6 months, $t(2)=22.26$, $p<0.02$ (Fig 1C2). No group differences emerged for the zero maze (Fig 2A).

No group differences emerged for the cued or spatial learning paradigms of the water maze (Fig 2B). However, jTBI animals exhibited fewer entries to the target area during the probe trials, $F(1, 13)=10.71$, $p<0.01$ (Fig 2C), suggesting a spatial memory dysfunction. Interestingly, the cumulative distance in the spatial learning is negatively correlated with the number of entries to the target area during the probe test ($r = -0.78$, $p<0.05$). This suggests that the animals with higher values for cumulative distance are less likely to enter in the target area during the probe test. The overall behavior changes at long term after jTBI are summarized in table 1.

Lesion Volume over time after jTBI

General characteristics of the injury were evaluated at 3 and 6 months. The original impact was administered at 17 days of age to the right fronto-parietal lobe (anterior somatosensory cortex), using a 3 mm diameter impactor centered over the cortex at bregma -1.0 mm. The approximate CCI site and resulting lesion cavity at 6 months is represented in Figure 3A–D. The underlying hippocampus was visible beneath the missing cortex by 6 months (arrow in Figure 3A) and the lesion cavity extended around the original CCI site (Figure 3B, D). An example of the lesion to brain volume is shown using a 3-dimensional reconstruction from a representative MR-scan (Figure 3C). The lesion spanned ~ 6 mm in length along the rostro-caudal axis, with an average distance from the frontal tip of the brain to the center of the cavity of 7.5 ± 0.2 mm. Overall, MR-derived lesion volumes at 3 and 6 months revealed that the percent lesion to brain volume remained stable over time in jTBI group at 3 months (mean % $\pm$ sem: 3.20 ± 0.43) and 6 months (mean % $\pm$ sem: jTBI 3.66 ± 0.62). The sham did not exhibit any visible lesion from 3 to 6 months.

Cortical Thickness

We compared the perilesional parietal cortical thickness to an identical region in the contralateral hemisphere using MR scans at 3 and 6 months. Representative MR-scans of coronal sections throughout the lesion site show decreased perilesional cortical thickness between jTBI and sham animals (Figure 4A), especially on the injured side of the brain closest to the lesion cavity. The groups did not have differences in contralateral cortical thicknesses at any *bregma* level ($p>0.1$). The average values for contralateral cortical thickness at 3 months were in jTBI: 1.74 ± 0.02 mm; sham: 1.74 ± 0.03 mm ($p>0.1$); at 6 months in jTBI: 1.61 ± 0.04 mm; sham, 1.57 ± 0.03 mm ($p>0.1$). In contrast, jTBI had significantly lower ipsilateral cortical thickness than sham at 3 months (group mean $\pm$ sem: jTBI, 1.49 ± 0.04 mm; sham, 1.68 ± 0.03 mm; $p<0.003$) and lower values were still evident at 6 months (group mean $\pm$ sem: jTBI, 1.41 ± 0.05 mm; sham, 1.53 ± 0.05 mm). The % ratio of ipsilateral/contralateral thickness was significantly lower in jTBI compared to sham across all 8 coronal levels using a repeated measure ANOVA ($p<0.05$,

Figure 4B, C). This data shows that jTBI cortical thickness was reduced by 0.1 – 0.2 mm compared to sham at several coronal levels, especially on the side closest to the lesion.

Corpus Callosum Thickness

The lesion cavity extended ventrally from the original impact site and often included portions of the CC, as we previously showed in another jTBI cohort at 60 days (Ajao, et al., 2012). We evaluated myelin basic protein (MBP) staining for visualization of white matter tracts (Figure 5 A, B), but did not detect any differences in staining intensity in the CC region (Figure 5C) at 6 months. However, the immunostaining revealed marked decreases in CC area on the side contralateral to the lesion (arrows in Figure 5B versus Figure 5A). Using MR-derived images at 3 months (Figure 5D, E, F) and 6 months (Figure 5G, H, I), we also show that jTBI animals have a thinner contralateral CC with a decrease of 27% at 3 months ($p < 0.001$) and 33% at 6 months ($p < 0.001$) compared to sham animals. The presence of long-term alterations to the CC may affect pathways involved in behavioral output.

Hippocampal Volume

The lesion extended along the longitudinal brain axis to encompass cortical areas above the hippocampus (Figure 3A–B), thus we performed a volumetric analysis of the ipsilateral and contralateral hippocampus at 3 and 6 months time points. At 6 months, a slight shift occurs in the position of the hippocampus along the different bregma levels in our analysis (see supplemental Figure 2), but, no significant changes were detected in the total hippocampal volume to explain the memory dysfunctions.

Tissue Characteristics

Histological samples were collected at 6 months and stained with the anti-MBP (Fig 5A, B), anti-NF200 (Fig 6), anti-NeuN antibody for neuronal nuclei (Figure 7) and anti-GFAP (Fig. 8).

The anti-MBP did not show any significant changes in intensity (Fig 5A, B), however significant structural changes were observed for the CC with a thinner thickness in jTBI group (Fig 5). This observation is confirmed with anti-NF200 staining showing a decrease of the CC thickness in injured group (Fig 6A, B). Anti-NF200 immunoreactivity shows decrease in intensity and changes in staining pattern in the ipsi and contralateral cortex (Fig 6 E, F), corpus callosum (Fig 6 I, J) and hippocampus (Fig 6 M, N) in jTBI group compared to the sham animals (Fig 6 C, D, G, H, K, L). Positive NF200 fibers are decreased in those regions and higher number of swollen neuronal cell bodies was observed in the contralateral cortex of the jTBI animals compared to the sham. All together, these data suggest long-term structural changes at distance of the original site of the impact (bregma –3.4 mm).

Images with NeuN staining revealed reduced cortical thickness near the lesion site compared to shams (Figure 7A, white arrowheads) and visibly structural changes of the hippocampus (Figure 7A, white arrow). NeuN labeling was also used for quantification of total NeuN positive neurons in the hippocampal CA1 region at bregma –3.4 mm (Figure 7B–C). Quantification revealed that jTBI animals have significantly less NeuN-positive cells in the CA1 field of the hippocampus in both ipsilateral and contralateral hemispheres to the lesion, (Figure 7B–C, H, I $p < 0.05$) in accordance with the DAPI counting (supplemental figure 3). To investigate possible ongoing delayed cell death at 6 months, activated caspase 3 were conducted in parallel to NeuN (Fig 7D, E, F, G). Few neuronal cells were positive for activated caspase 3 in both experimental group with no difference in total number (Fig 7D, E, F, G). Neuronal cell death in hippocampal CA1 was observed at 6 months, although the impact was administered in a region of parietal cortex far from the hippocampal formation

(Fig 3). Finally, astrogliosis was analyzed at 6 months time point showing no significant changes of the GFAP staining in the tissue (Fig 8) between jTBI and sham animals.

Discussion

Using a CCI model of experimental TBI in juvenile rats, we followed the outcome of an early-life injury (17 days of age) well into adulthood (6 months of age). Our data provide direct evidence that injury to the immature rodent brain results in long-lasting and continually evolving alterations in brain function and underlying structures. Notably, these effects are evident over a much longer post-injury timespan than is often experimentally employed. jTBI animals had persistent sensorimotor, coordination, and spatial memory deficits up to 6 months after injury. Over time, the initial impact evolved into a lesion cavity affecting both the parietal cortex and hippocampus. Collectively, our data indicate that jTBI in rats can be a useful model for studying long-term clinically-relevant behavioral dysfunction. Additionally, long-term evaluation of jTBI may prove relevant for neurodegenerative and pathological outcomes similar to brain aging (Pop and Badaut, 2011, Pop, et al., 2013) and provide avenues for testing the effects of protective and/or restorative drugs several months after the injury.

Differential characteristics of long-term behavioral outcomes

While most studies terminate TBI outcome evaluations at 2 or 3 months post-injury (Ajao, et al., 2012, Brody, et al., 2007, Prins and Hovda, 1998), the present study demonstrates differences for at least 6 months. In addition to the motor dysfunctions, we describe several behavioral deficits that are persistent, transient, or late-emerging in jTBI animals. Overall, our findings at 3, 5, and 6 months after injury are consistent with the literature corresponding with short-term injury outcome measures in both juvenile and adult animals (Ajao, et al., 2012, Fujimoto, et al., 2004, Hamm, et al., 1992, Hamm, et al., 1994, Hartman, 2011, Jones, et al., 2008)

Sensorimotor deficits were generally persistent over time, likely due to the original impact location in the right sensorimotor parietal cortex. jTBI animals demonstrated coordination and proprioception deficits compared to sham animals as evidenced by deficits on the foot fault task. These data are consistent with previously published data from 1 day to 2 months post-jTBI (Ajao, et al., 2012) and suggest that a focal injury in the right frontal cortex can have global effects on behavior. Specifically, jTBI animals demonstrated persistent sensorimotor and balance deficits on the rotarod, consistent with our study of the same injury model up to 2 months post-injury (Ajao, et al., 2012). Sensorimotor deficits appear to be a persistent characteristic following jTBI up to 6 months post-injury. The stable performance of both jTBI and sham groups further suggests that sensorimotor abilities are unlikely to change past 6 months. While an enriched environment or similar intervention could potentially benefit performance, the lack of improvements after multiple exposures to the rotarod suggests stable patterns of behavior, consistent with previous findings (Ajao, et al., 2012). On the foot fault task, however, coordination and proprioception differences resolved by 6 months post-injury. Additionally, jTBI rats were only impaired on the most difficult (fastest acceleration) rotarod paradigm, suggesting that sensorimotor and balance deficits are rather subtle and only observable during tasks with higher motor demands.

Besides the rotarod and foot fault, jTBI animals also demonstrated a persistent left turn bias during the water maze task for up to 6 months after injury. The direction of the turn bias was contralateral to the injury location (right frontal cortex), suggesting a left-sided hemiparesis. These results are also consistent with those that we demonstrated previously (Ajao, et al., 2012). Similar to the accelerating rotarod, this effect was persistent up to the end of the

protocol at 6 months and suggests long-lasting hemiparesis following a unilateral TBI sustained early in life.

Although jTBI animals did not demonstrate any learning deficits during the spatial trials of the water maze at any time point, we observed the emergence of spatial memory deficits at 3 months. Interestingly, these deficits were not observed either 1 or 2 months after injury (Ajao, et al., 2012, Pop, et al., 2013), highlighting the dynamic profile of jTBI effects beyond the time cutoffs often employed in behavioral research. The appearance of this effect at 3 months suggests that cognitive abilities previously viewed as “stable” throughout development can in fact degrade at the onset of young adulthood, and demonstrates the potential latent effects of a juvenile injury.

While spatial memory differences appeared to resolve by 6 months post-injury, it is plausible that increasing the difficulty of the water maze protocol would increase sensitivity, yielding differences at earlier or later time points. The protocol used in the present experiment was chosen based on previous data (Ajao, et al., 2012), and was kept the same across time points for statistical consistency. However, this paradigm may mask the learning effects of repeated testing. Since both sham and jTBI animals demonstrated similar spatial learning in our study (Fig 2B), using a more difficult protocol may be useful to further examine the nature, appearance, and resolution of memory deficits in jTBI.

Finally, our behavioral characterization of anxiety using the zero maze shows a fluctuating pattern of transient changes. Previous research with an identical injury model showed differences in anxiety-like behavior at 2 months post injury, with jTBI animals exhibiting more anxiety than shams (Ajao, et al., 2012). However, results in this study show that these differences are ameliorated by 3 months post injury, and no additional differences were observed up to 6 months post-injury (Fig 2A). This contrasts with an adult TBI model that showed anxiety-like behaviors up to 6 months after injury (Jones, et al., 2008). While repeated testing protocols and manipulating the animals may have mitigated affective differences in this study. In fact, it was shown by several groups that early postnatal stimulation handling (EH) produces profound and long-lasting behavioral and neurobiological effects (i.e. anxiety) and appears to induce “protective” effects against age-related cognitive deficits (Fernandez-Teruel, et al., 2002). More specifically measuring anxiety using elevated plus maze, the EH animals increase exploration of the open arms and time spent in illuminated compartment (Fernandez-Teruel, et al., 2002). Altogether these findings illustrate the difference in the long-term behavioral outcome between juveniles and adult animals.

Brain characteristics continue to evolve over time

Although lesion volume (normalized to brain volume) remained consistent over time, we observed several tissue changes beyond the original impact site (Fig. 3), that parallels the behavioral changes.

jTBI animals exhibited a percentage of lesion to brain volume of ~3% at all timepoints. However, we showed that the cavity expanded posteriorly to include areas beyond the original impact site (e.g., the hippocampus) and ventrally to disrupt other regions (e.g. corpus callosum) during brain development. In contrast with the 2 months time point (Ajao, et al., 2012) the morphological characteristics of the original impact evolved, reaching the posterior parietal cortex directly above the hippocampal formation and disrupting the integrity of communication pathways of the CC. The original impact at 17 days of age was centered at *bregma* –1.0 mm and administered using a 3mm diameter impactor. The resulting lesion cavity had a diameter of about 2 mm total and extended to approximately –3.0 mm posterior from *bregma* by 60 days (Ajao, et al., 2012). By 6 months, the lesion

diameter was about 6 mm and was visible on MR scans and post-fixation tissue from approximately +2.5 mm to -4.5 mm *bregma* (Fig 3 A–D). The ongoing recruitment of additional brain regions may explain the behavioral deficits observed on tasks involving multiple brain structures and pathways that are interconnected with the hippocampal formation.

Because the lesion appeared to change over time, we evaluated its possible effects on perilesional brain tissue (i.e., the parietal cortex, hippocampus, and corpus callosum). Using MR scans at 3 and 6 months and histological preparations at 6 months, we identified consistently reduced cortical thickness across several *bregma* levels. Interestingly, although the most anterior (+2.8 mm) and posterior (-4.8 mm) coronal levels did not contain a visible lesion cavity at 6 months, jTBI animals maintained lower cortical thickness ratio compared to sham animals at a distance from the center of the lesion site (Figure 4B–C). Not only was cortical thickness reduced on several coronal sections, but the lesion resulted in a disrupted CC on the ipsilateral side and a thinner CC tract in the contralateral hemisphere. In the same way, our observations of altered hippocampal position in the jTBI brain with MR scans (Fig. 4, supplemental fig 2) were consistent with the major changes observed in NF200, NeuN and DAPI labeling studies of the hippocampus (Figure 6, 7). In fact, NF200 analysis showed structural changes with less positive N200 positive fibers and swollen neurons in the cortex in the ipsi and contralateral. NF200 changes were also substantial in the hippocampus associated with less neurons in the CA1 layer in ipsi- and contralateral hippocampi at 6 months after injury, suggesting a long term neuronal cell death prior 6 months in this model. In fact, there is no difference in activated caspase 3 staining between the sham and jTBI animals suggesting the absence of significant apoptosis at 6 months (Fig 7). However, it does not exclude that apoptosis would occur earlier in the time. In contrast with early times points (Fukuda, et al., 2012), astrogliosis is not anymore observed around the cavity at 6 months post-TBI (Fig. 8).

Positive long-term histological findings contrast with a similar but less-severe injury model (Gobbel, et al., 2007), corroborating the idea of graded morphological and protein changes in response to an injury. Altogether, these observations identify possible avenues for further evaluation of long-term behavioral dysfunction and connections with distinct brain regions.

Conclusions

Experimental TBI in rodents can be used to model the long-term post-injury characteristics that are reported in the clinic. Given the increased incidence of TBI in young males, we evaluated several outcome measures using jTBI in male rats. Our data showed a progress in long-term behavioral changes following a single jTBI event, suggesting that jTBI is evolving in a chronic brain disorders. The pattern of observed results demonstrates persistent behavioral deficits in rats up to 6 months after juvenile brain injury that may have underlying structural brain correlates. While resolution of deficits was noted in some domains, others revealed persistent and stable deficits, which may be related to the recruitment of additional brain regions in the lesion.

Secondly, the changeable and transient nature of some deficits (primarily spatial memory) demonstrates that animals injured at a young age display a complex pattern of persistent, transient, and late-emerging cognitive and motor deficits observable into adulthood. Behavioral characteristics are still changing up to 6 months post injury, suggesting that the behavioral effects of TBI are dynamic and cannot be adequately captured using the 3-month terminus common in the literature.

Finally, our model suggests the need for continued evaluation after juvenile brain injury, and the development of rehabilitation programs directed at the improvement of long-term motor and memory functioning in clinical cases of jTBI. The emergence of long-term changes in several behavioral and structural domains may also indicate that neurodegenerative and pathological sequelae could underlie behavioral modifications similar to those seen in brain aging and disease post-trauma. Collectively, these changes can offer increased opportunities for obtaining therapeutic benefits even with delayed interventions. Future studies may include the use of therapeutic compounds to temper the behavioral deficits in jTBI animals, with the hope of developing a clinically-relevant treatment that can be used for pediatric TBI cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. A single juvenile TBI produced a chronic brain disorder with long-term complications
2. Persistent, transient, late-emerging behavioral deficits observable into adulthood.
3. Evolution of the initial lesion cavity during the brain development
4. Neuronal cell death in the hippocampus and white matter changes at distance to the initial site of the lesion.
5. A useful model for studying long-term behavioral and histological dysfunctions

Figure 1. Long-term sensorimotor deficits compared with shams

(A) jTBI animals exhibited motor coordination and balance deficits on the accelerating rotarod through the end of the experiment, up to 6 months post-injury. Performance on the rotarod for both jTBI and sham animals was stable over time. (B) jTBI animals exhibited motor coordination and proprioception deficits on the foot fault task up to 5 months post-injury. (C1) Sham animals exhibited no turn bias throughout the experiment. (C2) jTBI animals exhibited a persistent left turn bias at 3, 5, and 6 months post-injury. Variation bars are 95% confidence intervals.

Figure 2. Some cognitive differences, including spatial memory deficits, compared with shams (A) There were no differences in anxiety-like affective behavior between groups. (B) Both groups displayed the same rate of spatial learning on the water maze. (C) However, jTBI animals exhibited worse recall of spatial information up to 5 months post-injury.

Figure 3. The lesion evolves over at least 6 months

(A) A representative jTBI brain photographed post-PFA perfusion, shows the lesion as an open cavity spanning ~6 mm in length to reveal the hippocampal formation (black arrow) beneath the missing cortex. (B) A diagram of the rat skull showing the approximate location of the original CCI with respect to bregma, and the expansion of the lesion by 6 months. (C) A 3-dimensional reconstruction of the lesion and brain from a representative MR scan of a jTBI animal at 6 months. (D) A horizontal diagram of the rat brain showing the approximate location of the original CCI and the resulting lesion involving additional cortical areas, the corpus callosum, and the hippocampus. (CCI, controlled cortical impact; jTBI, juvenile traumatic brain injury; MR, magnetic resonance; PFA, paraformaldehyde; mm, millimeter)

Figure 4. Decreased cortical thickness

(A) At 6 months post-injury, representative MR-scans of several coronal sections throughout the lesion site show differences in cortical thickness (white arrowheads) between jTBI and sham animals, especially on the ipsilateral side of the lesion compared to the contralateral hemisphere. (B, C) MR-derived measures of cortical thickness are represented as the % ratio of ipsilateral/contralateral values for each individual animal, and no significant differences were detected between jTBI and sham in the contralateral side. There is a significantly lower ratio in jTBI animals than sham across bregma levels at both (B) 3 months and (C) 6 months. No significant differences were observed in cortical thickness among groups between the 3 and 6 month time points. (jTBI, juvenile traumatic brain injury; MRI, magnetic resonance imaging; mm, millimeter; % I/C, ratio ipsilateral/contralateral; Cx Thk, cortex thickness)

Figure 6. Altered NF200 labeling in jTBI

(A) At 6 months post-injury, representative NF200 immunoreactivity at bregma -3.8 mm coronal sections in sham shows the cortex (Cx), corpus callosum (CC) and hippocampus. (B) At similar coronal level, the NF200 labeling is markedly decreased in the hippocampus (arrows) located under the cavity (asterisk). (C, D, E, F) NF200 staining in the parietal cortex adjacent to the site of the impact (E, ipsi) in jTBI and equivalent region in sham (C, ipsi) and contralateral parietal cortex (contra) in sham (D) and jTBI (F). The overall intensity of the staining is decreased with less visible NF200-positive fibers in ipsi and contralateral cortex of jTBI animals (E, F) compared to sham (C, D). (G, H, I, J) NF200

staining in the corpus callosum (CC) in the sham (G, H) and jTBI (I, J) show overall changes in the pattern of staining with less fibers and fainter intensity in jTBI group. (K, L, M, N) NF200 staining in the hippocampus, CA1 region for sham (K, L) and jTBI (M, N) shows less neuronal fibers in stratum radiatum (SR). Similar to NeuN staining (Fig 7), CA1 layer is thinner with likely less neuronal cell bodies in CA1 of the jTBI rats (M, N) compared to the sham (K, L).

Bar: A, B= 500 μ m; C–N= 100 μ m

Figure 7. Neuronal cell death in hippocampus

(A) Histological evaluation at 6 months post-injury shows the anti-NeuN antibody and an infra-red (IR) secondary antibody (wavelength 800nm), for visualization of general tissue structure. Sections corresponding to bregma -3.4 mm and -5.8 mm are shown for a representative sham and jTBI. Notably, qualitative differences were observed in several jTBI animals, namely reduced cortical thickness at coronal levels near the lesion site (white arrowheads) and reduced hippocampal volume compared to shams (white arrow). (B, C) Sections were labeled with NeUN in CA1 of sham (B) and jTBI animals (C) show decreased of number positive neurons in contralateral hippocampus. (D, E) Activated caspase-3

showed some positive staining in hippocampus of the sham (E) as well as in jTBI animals (D). **(F, G)** the merge NeuN (green) and caspase-3 (red) showed co-localization in yellow (arrow), showing presence of activated caspase-3 in hippocampus of both groups. **(H, I)** Counting of positive NeuN neurons in ipsilateral CA1 (H) and contralateral CA1 (I) showed a significant decrease in numbers (*, $p < 0.05$). Bar B–G = 50 μm .

Figure 8. Absence of significant astrogliosis around the cavity

(A) Double immunohistochemistry NeuN (red) and GFAP (green) co-stained with DAPI (blue) in the parietal cortex adjacent to the lesion (cavity, arrows) did not show any major astrogliosis at 6 month post injury. The NeuN staining shows a thinner CA1 layer despite high GFAP staining. (B, C) GFAP staining in the sham (B) and jTBI (C) did not show any major difference in the cortex (Cx), corpus callosum (CC) and hippocampus located under the cavity (C, *). (D, E) Higher magnification in the parietal cortex adjacent to the cavity in the jTBI group (E) did not show higher GFAP staining (arrow) compared to the sham group (D).

Bar is A= 500 μ m; B–C =1mm; D–E=100 μ m

Table 1

Summary of the behavior changes.

	3 Month Group Differences?	5 Month Group Differences?	6 Month Group Differences?	jTBI Summary
Rotarod	Yes	Yes	Yes	Persistent deficits
Foot Faults	Yes	Yes	No	Deficits resolve by 6 months post-injury
Turn Bias	Yes	Yes	Yes	Persistent left bias
Zero Maze	No	No	No	No differences
Sp. Learning	No	No	No	No differences
Sp. Memory	Yes	Yes	No	Memory differences resolve by 6 months post-injury