Traumatic Injury to the Immature Brain Results in Progressive Neuronal Loss, Hyperactivity and Delayed Cognitive Impairments

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Key Words
Traumatic brain injury · Immature brain · Cognitive function · Delayed cell loss

Abstract
The immature brain may be particularly vulnerable to injury during critical periods of development. To address the biologic basis for this vulnerability, mice were subjected to traumatic brain injury at postnatal day 21, a time point that approximates that of the toddler-aged child. After motor and cognitive testing at either 2 weeks (juveniles) or 3 months (adults) after injury, animals were euthanized and the brains prepared for quantitative histologic assessment. Brain-injured mice exhibited hyperactivity and age-dependent anxiety. Cortical lesion volume and subcortical neuronal loss were greater in brain-injured adults than in juveniles. Importantly, cognitive decline was delayed in onset and coincided with loss of neurons in the hippocampus. Our findings demonstrate that trauma to the developing brain results in a prolonged period of pathogenesis in both cortical and subcortical structures. Behavioral changes are a likely consequence of regional-specific neuronal degeneration.

Introduction
Cognitive dysfunction and disruption of attention and information processing ability are common sequelae of traumatic brain injury in children [Levin et al., 1982; Murray et al., 1992]. Neurobehavioral sequelae in brain-injured children include attention deficit and hyperactivity disorder [Konrad et al., 2003], as well as impairments in learning, memory and executive functions [Anderson et al., 1996; Ewing-Cobbs et al., 1989; Levin et al., 1998; Yeates et al., 1995].

The immature brain may be vulnerable to injury during critical periods of development. Children less than 4 years of age exhibit poorer motor and cognitive function than older children after traumatic brain injury [Ewing-Cobbs et al., 1989; Koskineni et al., 1995; Luerssen et al., 1988]. Approximately 20% of these young children attain a favorable outcome after traumatic brain injury, as defined by good recovery and moderate disability, by 1 year after injury [Ewing-Cobbs et al., 1989]. This is in sharp contrast to older children where approximately 75% attain a favorable outcome in a similar time frame.

Poor outcomes may be attributed to a failure to meet new developmental demands [Radcliffe et al., 1994].Brain injury at a young age interferes with acquisition of cognitive skills, and hence, the young child may initially appear to have few observable deficits. However, impairments
may emerge with time, as the impact of poor skill acquisition results in increasing discrepancies between the brain-injured child relative to age-matched peers. These latent deficits may be realized as an inability to carry out new skills or engage in a specific cognitive process, as opposed to a slowed rate of development or a cumulative impairment in functioning [Taylor and Alden, 1997].

We developed a murine model of traumatic injury to the immature brain [Tong et al., 2002] in an effort to better understand the persistent behavioral sequelae, progressive decline and delayed emergence of cognitive deficits following traumatic brain injury to young children [Crack et al., 2001; Levin et al., 1998]. In this model, mice are subjected to traumatic brain injury at postnatal day 21, an age that is roughly equivalent to the toddler-aged child [Rice and Barone, 2000; Yager and Thornhill, 1997]. We selected controlled cortical impact for these developmental studies as it reproduces many of the pathologic sequelae that have been reported in traumatic brain-injured children including focal contusions, subdural hematomas and diffuse axonal injury [Dixon et al., 1991; Graham et al., 1989; Hahn et al., 1988; Michaud et al., 1993]. In our experimental model, cortical impact to the frontoparietal cortex results in a predictable pattern of both cortical and subcortical neuronal loss by 2 weeks after injury. A focal injury is produced in the cortex and there is significant neuronal loss in the CA3 regions of the hippocampus and dorsal thalamus [Tong et al., 2002]. In the present study, this model was used to study early (2 weeks) and long-term (3 months) consequences of traumatic brain injury on motor and cognitive function and to determine whether they are associated with progressive cortical and subcortical neuronal loss during brain maturation.

Materials and Methods

Animal Model

All experimental procedures were institutionally approved in accordance with the University of California and Oregon Health and Science University Committees on Animal Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice at postnatal day 21 were anesthetized with 1.25% (w/v) 2,2,2-tribromoethanol (Avertin, 0.02 ml/g body weight, i.p.) and subjected to traumatic brain injury (n = 20) or sham surgery (n = 20) as previously described [Tong et al., 2002]. The anesthetized animal was placed on a circulating water heating pad to maintain normal body temperature (36.5–37.5°C) throughout the surgical period and during the postoperative period until full recovery from the anesthesia. In each animal, the scalp was opened with a midline incision, and a circular craniotomy, 5.0 mm in diameter, was made over the left parietal region between bregma and lambda with the medial edge of the incision 0.5 mm lateral to the midline. The head was then positioned in the stereotaxic frame of the injury device and subjected to a controlled cortical impact insult, using a 3.0-mm convex impactor tip oriented perpendicular to the cortical surface. The injury parameters were set as follows: 4 m/s velocity, 1 mm depth of penetration and 150 ms dwell time. Care was taken to maintain the dura intact. The skin, overlaying the site of the craniotomy, was sutured. Each animal was administered physiologic saline (1.0 ml, s.c.) prior to surgery and again during recovery from anesthesia in order to maintain adequate hydration. Sham-operated controls consisted of the same surgical procedures but were not subjected to traumatic injury.

Brain-injured animals and sham-matched age controls were subjected to behavioral evaluations beginning at either 2 weeks (juvenile cohort) or 3 months after injury (adult cohort). At the completion of the behavioral evaluations, each animal was euthanized, perfused as described below, and the brain removed and prepared for histologic assessment.

Behavioral Evaluations

An extensive battery of behavioral tests was performed on sham and brain-injured mice. The sequence of behavioral testing was administered in order of increasing stress levels. The order of testing was as follows: open field, elevated plus maze, elevated zero maze (week 1), novel object recognition (week 2), rotordor, balance beam, wire hang, inclined screen (week 3) and water maze (week 4). After each of these tests, except for the water maze, the equipment was cleaned with 1 mM acetic acid to remove residual odors that could affect testing of subsequent animals. All behavioral tests were conducted the same way for both cohorts. The experimenter was blinded to the treatment of the animals.

Open Field. Since different levels of exploratory drive and anxiety can affect motivation and performance in cognitive tests, these functions were evaluated first. Exploratory behavior was assessed in the open field, and measures of anxiety were evaluated in the open field, elevated plus maze and elevated zero maze.

Mice were placed singly in a brightly lit, automated infrared photocell activity arena (40.64 × 40.64 cm, with 16 × 16 photocells for measuring horizontal movements and 8 photocells for measuring rearing) interfaced with a computer (Hamilton-Kinder, Poway, Calif., USA). The open field was divided into a center zone (20.32 × 20.32 cm) and a peripheral zone. The following parameters were calculated: active times (defined as time, within 1 s, in which a new beam was broken), distance moved, rearing events, periphery entries, center entries and percentage of time spent in the center zone. Open field activity was recorded after a 1-min adaptation period for 10 min. Increases in center zone measures are thought to reflect decreased measures of anxiety [Lister, 1990].

Elevated Plus Maze. The elevated, plus-shaped maze consists of two open arms and two closed arms equipped with rows of infrared photocells interfaced with a computer (Hamilton-Kinder). Rats avoid the open arms of the plus maze so that decreased time spent in and decreased entries into the open arms are thought to reflect enhanced measures of anxiety [Lister, 1990]. Mice were placed individually in the center of the maze and allowed free access for 10 min. Animals spent time either in a closed, safe area (closed arms), in an open area (open arms) or in the middle, intermediate zone. Recorded beam breaks were used to calculate the

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time spent and the distance moved in the open and closed arms and the number of times the mice reached over the edges of the open and closed arms.

**Elevated Zero Maze.** The elevated zero maze (Hamilton-Kind er) consists of two enclosed areas and two open areas and has a diameter of 53.34 cm. The open and closed areas are identical in length to the open and closed arms in the elevated plus maze. Mice were placed in the closed part of the maze and allowed free access for 10 min. A Noldus video tracking system was used to calculate the time spent in the open areas.

**Novel Location and Novel Object Recognition.** Novel location/ novel object recognition was used to evaluate hippocampal- and cortical-dependent nonspatial learning and memory. On 3 consecutive days, mice were habituated to an open field for 5 min. On the fourth day, mice were trained in three consecutive trials and then tested in two consecutive trials with a 5-min intertrial interval. For the training and testing sessions, three different plastic toys were placed in the open field, and the animal was allowed to explore for 10 min. All objects were used only once. Replicas of the objects were used in subsequent trials to eliminate potential confounds of residual odors or potential scratching marks on particular objects. In the first test trial, one of the familiar objects was moved to a novel location. In the second test trial, one of the familiar objects was replaced by a novel object. For all trials, the time spent exploring each object was recorded and the percentage of time spent exploring each object was calculated.

**Rotorod.** Rotorod balancing requires a variety of proprioceptive, vestibular and fine-tuned motor abilities. The task requires the mouse to balance on a rotating rod (Hamilton-Kinder), which is 7.0 mm in diameter and is used to screen for sensorimotor deficits [Rustay et al., 2002]. After a 1-min adaptation period on the rod at rest, the rod was accelerated by 2 rpm every 30 s, and the length of time the mice remained on the rod (fall latency) was recorded.

**Inclined Screen and Beam Balance.** To determine potential impairments of balance, mice were tested in the inclined screen and balance beam tests. In the inclined screen test, mice were placed on a 182-cm-long inclined screen (36° incline), 128.5 cm from the top and 35.3 cm from the bottom of the screen, and were allowed to explore for 3 min. Mice have a natural propensity to climb upwards. The total distance moved and the mean velocity of movement were recorded with a Noldus Instruments Ethovision video tracking system. The event recorder of the Noldus software was used to determine the frequency and duration of missteps, defined as placing any paw between the grids of the screen (the diameter of the rods on the grid was 3 mm, and the distance between the rods was 17 mm).

In the balance beam test, mice were placed in the middle of a horizontal beam (88.5 cm long, 1.8 cm in diameter) 30 cm above a horizontal surface. The total distance moved and the mean velocity of movement in two trials of 2 min each were recorded with a Noldus Instruments Ethovision video tracking system.

**Wire Hanging.** To evaluate muscle strength, mice were lifted up by their tails and slowly placed on a horizontal cotton wire (1 mm in diameter) allowing them to use both fore- and hindpaws to grab the wire. The placing procedure takes 1–2 s and does not require pretrained or habituation. The wire was mounted 60 cm above a horizontal surface. Once the mice attached to the wire, it was a little bent out of the horizontal plane and slightly V shaped. Two sequential trials were given, and the length of time that the mice held onto the wire (latency) was recorded.

**Water Maze Learning.** To assess spatial learning and memory requiring navigation to a target, mice were trained to locate a hidden submerged platform in a pool (diameter 122 cm) filled with warm (24°C) opaque water. To find the platform, mice had to relate their position in the pool to constant extramaze cues and then quickly store, retrieve and utilize that information to determine where the platform was located. Mice were placed into the water facing the wall at the side of the pool in nine different locations around the pool circumference. The starting location was changed for each trial. Mice were trained to first locate a visible platform (days 1 and 2) and then a submerged hidden platform (days 3–5) in two daily sessions 3.5 h apart; each session consisting of three 60-second trials (at 10-min intervals). Mice that failed to find the hidden platform within 60 s were manually placed on it for 15 s. For data analysis, the platform was divided into four quadrants. During the visible platform training, the platform was moved to a different quadrant for each session. During the hidden platform training, the platform location was kept constant for each mouse (in the center of the target quadrant). The starting point at which the mouse was placed into the water was changed for each trial. Time to reach the platform (latency), path length and swim speed were recorded with the Noldus Ethovision video tracking system set to analyze two samples per second. A 60-second probe trial (platform removed) was performed 1 h after the last hidden platform session.

**Immunocytochemistry**

Following behavioral testing, at 5 weeks after injury (juvenile cohort) and 4.5 months after injury (adult cohort), animals were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed, fixed in 4% paraformaldehyde and cryoprotected in sucrose (20% in PBS). The cryoprotected brains were embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, N.C., USA) and frozen at −70°C. Frozen sections, 50 μm in thickness, were cut on a sliding microtome in the coronal plane and collected as free floating sections in wells containing 0.05 M PBS. Sections of brain were processed for conventional immunocytochemistry. Every sixth section was immunostained for NeuN (1:1,000 dilution; Chemicon International, Temecula, Calif., USA). The antibody was prepared in blocking solution (MOM kit, Vector Laboratories, Burlingame, Calif., USA) to avoid nonspecific background staining. Sections were incubated with the primary antibody for 30 min at room temperature. The secondary antimouse antibody (Vector Laboratories) was used at a 1:250 dilution. Final detection of the signal using a biotinylated derivative (Vector Laboratories) was visualized with a peroxidase substrate. Immunocytochemical controls consisted of omission of the primary antibody. Images were captured using Neurolucida 5.0 (MicroBrightField, Inc., Williston, Vt., USA) and Photoshop 6.0 (Adobe Systems, San Jose, Calif., USA).

**Regional Cell Counting and Estimation of Lesion Volume.** Cell counting and estimation of lesion volume were conducted in a blinded fashion. The relative number of NeuN-labeled neurons was determined on three coronal sections from each brain (n = 8 per group for each cohort). These sections were each separated by 300 μm and were centered over the maximal site of injury. The relative number of neurons was determined within CA3 of the hippocampus and the dorsal thalamus, subcortical regions that have been previously shown to be vulnerable after traumatic brain injury in both the adult and developing brain [Igarashi et al., 2001;
working concentrations: goat antidoublecortin (diluted 1:200; Palmer et al., 2000) using the following primary antibodies and techniques as previously described [Palmer et al., 2000]. Appropriate gain and black-level settings were obtained on control tissues stained with secondary antibodies alone. Upper and lower thresholds were always set using a range indicator function to minimize data loss due to saturation. The threshold was determined with both traumatic brain injury and sham surgery groups. The primary confocal endpoint was the total number of doublecortin-positive cells. Cell counts, conducted in a blinded fashion, were limited to the dentate granular cell layer and a 50-μm border along the hilus margin that included the subgranular zone. Each cell was manually examined in its full z-dimension with use of split panel analysis, and only those cells for which the doublecortin-positive processes were unambiguously associated with nuclei were scored as positive. All positively labeled cells within the subgranular zone of the superior and inferior blades of the dentate gyrus in both hemispheres were counted on each section. Values from both sections were subsequently summed for each animal.

**Statistical Analyses**

Data were expressed as mean ± SEM. Data were analyzed by ANOVA for main effects of cohort (juvenile or adult) and treatment and for cohort × treatment interaction, followed by Dunnett’s or Tukey-Kramer posthoc tests when appropriate. Learning curves were compared by repeated-measures analysis of variance using contrasts to assess differences between specific groups of mice. For correlation analyses, Pearson correlation calculations were performed. Unpaired t tests were used to compare sham and injured groups at each of the time points. In all experiments, p < 0.05 was considered significant.

**Results**

**Traumatic Brain Injury Increases Exploratory Activity and Decreases Anxiety Levels**

When exploratory activity was evaluated in a novel open field, brain-injured mice showed higher total activity levels than sham controls (F = 0.837; d.f. = 1; p = 0.0095) (fig. 2A). There was also a difference between the exploratory levels of juvenile and adult cohorts (F = 4.495; d.f. = 1; p = 0.0410), but there was no cohort by treatment interaction (F = 0.001; d.f. = 1; p = 0.9810). For analysis of measures of anxiety, the open field (40.64 × 40.64 cm) was divided into a center zone (20.23 × 20.23 cm) and a peripheral zone. Increases in center zone measures are thought to reflect decreased measures of anxiety [Lister, 1990]. There were no differences between the injured and sham control mice in either cohort in total time and rest time spent in the center zone, indicating that injury did...
not alter measures of anxiety in the open field. As observed for the total arena, brain-injured mice showed higher activity levels than sham controls in the peripheral zone (F = 4.970; d.f. = 1; p = 0.0321) (fig. 2A), and there was a difference between the exploratory levels of the juvenile and adult cohorts (F = 6.995; d.f. = 1; p = 0.0120).

Differences in activity levels were also evident when performance was evaluated in the elevated plus maze (fig. 2B). Brain-injured mice entered the intersection more frequently than sham controls (F = 5.228; d.f. = 1; p = 0.0282). There was a difference in entries into the closed arms between the juvenile and adult cohorts (F = 6.751; d.f. = 1; p = 0.0135) and an interaction between cohort and treatment (F = 14.700; d.f. = 1; p = 0.0369); there was an effect of treatment in the adult (p = 0.025), but not the juvenile, cohort (p = 0.473). These differences in activity levels in the plus maze confounded our ability to use open arm measures as measures of anxiety. Thus, the higher open arm entries in brain-injured mice, relative to shams (F = 5.799; d.f. = 1; p = 0.0213), might simply reflect effects of injury on activity levels. Therefore, in the elevated zero maze, we evaluated the percentage of time the mice spent in the open areas (fig. 2), a measure not affected by the potential confound of differences in activity levels. There was a difference in the percentage of time spent in the open areas of the zero maze between brain-injured mice and sham controls (F = 6.109; d.f. = 1; p = 0.0185) (fig. 2); in the juvenile cohort, brain-injured mice showed reduced measures of anxiety as compared with sham-injured controls (p = 0.010). This was not seen in the adult cohort (p = 0.463). In the elevated zero maze, measures of anxiety were also lower in the juvenile cohort than in the adult cohort (F = 26.623; d.f. = 1; p < 0.0001).

No Effects of Traumatic Brain Injury on Sensorimotor Function

Sensorimotor function was assessed using a rotord, which requires a combination of muscle tone and sensory input (fig. 3). All groups exhibited improvement with training (F = 25.543; d.f. = 8; p < 0.0001). However, there were no treatment differences in rotord performance (F = 0.116; d.f. = 1; p = 0.6163). Next, potential differences in muscle strength were assessed with the wire hang test, and potential differences in balance were assessed with the balance beam and inclined screen tests. While the mice in the juvenile cohort were able to maintain the position on the wire longer than mice in the adult cohort (F = 46.745; d.f. = 1; p < 0.0001), there were no differences between brain-injured and sham controls in either
cohort (fig. 3). On the balance beam, juvenile mice also moved more ($F = 4.466$; d.f. = 1; $p = 0.0416$) and faster ($F = 33.872$; d.f. = 1; $p < 0.0001$) than adult mice, but there were no differences between brain-injured mice and sham controls in either cohort in distance moved ($F = 0.005$; d.f. = 1; $p = 0.9448$) or velocity ($F = 0.101$; d.f. = 1; $p = 0.7527$) (table 1). In the inclined screen test, there were no effects of treatment or cohort (data not shown). The improved performance of brain-injured and sham controls in juveniles in the wire hangs and balance beam tests could be due to the relatively younger age of these mice.

**Table 1.** Performance of brain-injured mice and sham controls on the balance beam

<table>
<thead>
<tr>
<th>Treatment/cohort</th>
<th>Distance moved, cm</th>
<th>Velocity, cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>trial 1</td>
<td>trial 2</td>
</tr>
<tr>
<td>Sham/juvenile</td>
<td>157 ± 28</td>
<td>162 ± 29</td>
</tr>
<tr>
<td>TBI/juvenile</td>
<td>123 ± 28</td>
<td>160 ± 22</td>
</tr>
<tr>
<td>Sham/adult</td>
<td>117 ± 27</td>
<td>77 ± 28</td>
</tr>
<tr>
<td>TBI/adult</td>
<td>121 ± 17</td>
<td>101 ± 31</td>
</tr>
</tbody>
</table>

Mice were tested in two subsequent trials. While there was an effect of cohort there was no effect of treatment in either cohort (see text).

Values are expressed as means ± SEM (n = 10 mice per treatment in each cohort). TBI = Traumatic brain injury.

**Fig. 3.** Sensorimotor function in traumatic brain-injured (TBI) and age-matched sham controls. Sensorimotor function and muscle strength were assessed using the rotorod (**A**) and wire hang test (**B**), respectively. **A** There were no group differences in rotorod performance. **B** Both injured and sham-matched controls in the juvenile cohort showed higher wire hang fall latencies than both groups in the adult cohort ($p < 0.01$, Tukey-Kramer). There was no significant difference in fall latency between injured animals and age-matched sham controls within either cohort. Values are expressed as means ± SEM (n = 10 mice per treatment in each cohort).

**Fig. 4.** Spatial learning and memory in traumatic brain-injured (TBI) animals and age-matched sham controls. **A** Time to locate the visible (sessions 1–4) and hidden (sessions 5–10) platform locations (latency) during water maze training are shown. Injured animals and age-matched sham controls in both cohorts did not show any significant difference in their ability to locate the visible platform. However, injured animals in the adult cohort showed higher latencies to locate the hidden platform than age-matched sham controls ($p < 0.05$, Tukey-Kramer). **B** All groups showed memory retention and spent more time in the target than in any other quadrant. * $p < 0.05$ and ** $p < 0.01$ versus any other quadrant, Dunnett’s test. Values are expressed as means ± SEM (n = 10 mice per treatment in each cohort).
When hippocampal/cortex-dependent object recognition was evaluated, there were no differences between brain-injured and sham controls in total time spent exploring objects over five trials or in object recognition (data not shown). Next, spatial learning and memory requiring navigation to a target were assessed in the water maze. As there were no differences in swim speeds during the visible sessions, time to locate the platform (latency) could be used as performance measure during acquisition (fig. 4). While brain-injured animals and sham controls did not differ in their ability to locate the visible platform, they differed in their ability to locate the hidden platform ($F = 4.606; \text{d.f.} = 1; p = 0.0391$), and there was an interaction between treatment and session during hidden platform training ($F = 26.695, \text{d.f.} = 5, p < 0.0001$; effect of session in each treatment groups: $p < 0.0001$).

The impairment in acquisition during hidden water maze training but intact memory retention in the subsequent probe trial might relate to the fact that while the brain-injured animals were impaired in acquisition compared with sham controls, both treatment groups showed improvement in locating the hidden platform with training (overall effect of session: $F = 26.695, \text{d.f.} = 5, p < 0.0001$; effect of session in each treatment groups: $p < 0.0001$).

**Traumatic Brain Injury Induces Age-Dependent Spatial Learning Deficits**

Fig. 5. Cortical lesion volume expands over time after traumatic brain injury. A Cortical cavitation involves the frontal and parietal gray matter and subcortical white matter at 5 weeks after injury (juvenile cohort). B There is a marked expansion of the cortical cavitation at 4.5 months after injury (adult cohort). C, D Stereological tracings of total brain volume and lesion volume in injured animals in the juvenile cohort (C) and adult cohort (D). Scale bar = 1.0 mm. E There is a significant increase in the ratio of lesion volume in injured animals in the adult cohort relative to the juvenile cohort. Values are expressed as means ± SEM ($n = 7$ mice per cohort). * $p = 0.0488$, unpaired t test.

E There is a significant increase in the ratio of lesion volume in injured animals in the adult cohort relative to the juvenile cohort. Values are expressed as means ± SEM ($n = 7$ mice per cohort). * $p = 0.0488$, unpaired t test.
Cortical Lesion Expands during Brain Maturation

Sections from sham-operated controls did not reveal any apparent lesion in either cohort (data not shown). In brain-injured animals, a cortical lesion involving somatosensory and motor cortex was apparent in both cohorts (fig. 5A–D). This cortical lesion typically incorporated the adjacent lateral ventricle and extended from the retrosplenial to the visual cortices in both cohorts. In the adult cohort, the cortical lesion appeared to occupy a greater proportion of the sensory, motor and visual cortices.

There were no differences in total brain volume between each of the cohorts. However, lesion volume increased from 0.0216 ± 0.0051 (mean ± SEM, unpaired t test) in the juvenile cohort to 0.0464 ± 0.0108 (mean ± SEM, unpaired t test) in the adult cohort. Similarly, there was an increase in the ratio of lesion volume to total brain volume in the adult cohort relative to the juvenile cohort (p = 0.0488, unpaired t test) (fig. 5E). These findings suggest that the cortical lesion continues to expand during brain maturation.

Relative Neuronal Density Decreases in the Hippocampus and Thalamus after Injury

There was overt neuronal loss in the CA3 region of the hippocampus and laterodorsal thalamus after brain injury in each of the cohorts (fig. 6A–F, 7A–F). This neuronal loss appeared to be more profound within each of these subcortical regions in the adult cohort as compared with the juvenile cohort (fig. 6A–D, 7A–D).

Relative neuronal density was determined in the CA3 region of the hippocampus (fig. 6E, F) and in the laterodorsal thalamus (fig. 7E, F). Neuronal density in CA3
and the thalamus was similar in sham-operated, juvenile animals as compared with sham-operated, adult animals. Neuronal density was reduced in these subcortical regions in brain-injured animals in both cohorts as compared with their respective age-matched sham controls (juvenile cohort: \( p = 0.0003 \); adult cohort: \( p < 0.0001 \); unpaired \( t \) tests). Neuronal density was also reduced in CA3 and the thalamus of brain-injured animals in the adult cohort as compared with brain-injured animals in the juvenile cohort (CA3: \( p = 0.0134 \); thalamus: \( p = 0.0258 \); unpaired \( t \) tests) (fig. 6F, 7F). These findings suggest that neuronal loss in CA3 and the thalamus is not restricted to the first several weeks after injury but may rather proceed over a more extended time course during brain maturation.

**Immature Neurons Are Reduced in the Subgranular Zone after Traumatic Brain Injury**

We next determined if the vulnerability of the hippocampus to traumatic injury would be reflected in the population of immature neurons that reside in the subgranular zone. These immature neurons were defined in brain sections that had been immunolabeled with doublecortin, a tubulin-associated protein expressed in migrating neuroblasts [Jessberger and Kempermann, 2003; Kempermann et al., 2003; Mizuguchi et al., 1999; Nacher et al., 2001] (fig. 8A–F). Several qualitative differences were noted in immunostained sections. First, there appeared to be fewer labeled cells in the ipsilateral and contralateral dorsal thalamus of sham controls in the juvenile cohort as compared with the adult cohort. F There is a significant reduction in the relative density of neurons in the ipsilateral dorsal thalamus in brain-injured animals in the juvenile cohort as compared with the adult cohort. * \( p = 0.0258 \), unpaired \( t \) test. Values are expressed as means ± SEM (\( n = 8 \) mice per treatment in each cohort).

Fig. 7. Relative density of NeuN-positive neurons is reduced in the dorsal thalamus after traumatic brain injury. A, B Age-matched sham controls in juvenile (A) and adult (B) cohorts. C Typical appearance of the dorsal thalamus at 5 weeks after injury (juvenile cohort). D There appear to be fewer neurons in the dorsal thalamus by 4.5 months after injury (adult cohort) as compared with the juvenile cohort. Scale bar = 0.1 mm. E There is no difference in the relative density of neurons in the ipsilateral and contralateral dorsal thalamus of sham controls in the juvenile cohort as compared with the adult cohort. F There is a significant reduction in the relative density of neurons in the ipsilateral dorsal thalamus of brain-injured animals in the adult cohort as compared with the juvenile cohort. * \( p = 0.0258 \), unpaired \( t \) test. Values are expressed as means ± SEM (\( n = 8 \) mice per treatment in each cohort).
There is an age-dependent change in the number of doublecortin-positive neurons in the dentate gyrus after traumatic brain injury. A–F Doublecortin-positive immature neurons in the dentate gyrus of age-matched sham controls (A–B) and brain-injured animals (C–F). The pattern of immunolabeling appears qualitatively similar in the injured ipsilateral dentate gyrus (C) relative to the ipsilateral, age-matched sham in the juvenile cohort (A). In contrast, there appear to be fewer labeled cells in the injured ipsilateral dentate gyrus (D) relative to the ipsilateral age-matched sham in the adult cohort (B). The contralateral dentate gyrus for brain-injured animals in juvenile and adult cohorts is shown in E and F, respectively. Scale bar = 50 μm. G There is no difference in the number of doublecortin-labeled neurons in the ipsilateral dentate gyrus of brain-injured animals relative to sham controls in the juvenile cohort. H There is a significant reduction in the number of neurons in the ipsilateral dentate gyrus of brain-injured animals relative to sham controls in the adult cohort. * p = 0.002. Values are expressed as means ± SEM (n = 5 mice per treatment in each cohort).
controls in the adult cohort (p = 0.002, unpaired t test) in injured animals as compared with age-matched sham labeled cells in the ipsilateral subgranular zone in brain-matched controls (fig. 8G). In contrast, there were fewer injured, juvenile animals as compared with their age-labeled cells in the ipsilateral subgranular zone in brain-injured animals as compared with age-matched sham controls in the adult cohort (p = 0.002, unpaired t test) (fig. 8H).

Discussion

Toddler-aged children are particularly vulnerable to traumatic brain injury as evidenced by persistent neurological impairment [Durkin et al., 1998]. We have begun to examine the underlying substrates that may define this vulnerability using an experimental model of traumatic injury to the developing murine brain [Tong et al., 2002]. We present the first evidence that traumatic injury to the frontoparietal cortex at postnatal day 21 results in hyperactivity, age-dependent decrease in measures of anxiety and spatial learning deficits. Whereas hyperactivity is evident by 2 weeks after injury, learning deficits are delayed in onset and are not detected until early adulthood. In contrast, anxiolysis is seen at 2 weeks after injury but is no longer present in early adulthood. We further show enlargement of the cortical lesion, progressive loss of neurons within the CA3 region of the hippocampus and fewer doublecortin-positive neuroblasts in the dentate gyrus during brain maturation. This extended period of pathogenesis may underlie the observed behavioral abnormalities including cognitive decline.

Our findings have important clinical implications as they suggest that poor recovery after trauma to the developing brain may be related to the evolution of injury. Thus, extending the window of therapeutic intervention for the brain-injured child may result in greater restoration of function.

Hyperactivity and Age-Dependent Anxiolysis after Traumatic Brain Injury

Hyperactivity was evident in brain-injured mice. Mice were more active in a novel environment following brain injury as compared with age-matched sham controls. Brain-injured mice also exhibited age-dependent anxiolysis. Reduced measures of anxiety were apparent in the juvenile cohort, relative to sham controls, but were not evident in the adult cohort.

Clinical studies demonstrate similar findings. Attention deficit/hyperactivity disorder is one of the most common psychiatric disorders after pediatric traumatic brain injury [Bloom et al., 2001; Konrad et al., 2003]. This syndrome is associated with a varied pattern of symptom clusters and neurocognitive correlates including difficulties with attention, motor overactivity and impulsivity that interfere with normal function [Max et al., 2003]. Anxiety disorders have also been reported after pediatric traumatic brain injury in humans [Vasa et al., 2004], as well as in other animal models of brain injury [Lacroix et al., 2000; Rangel et al., 2003; Shah and Treit, 2003].

The determinants of hyperactivity and anxiolysis after traumatic brain injury are unclear and may be related to the extensive cortical lesion that includes the retrosplenial cortex, a region that is richly connected to the prefrontal cortex by both afferents and efferents [Zilles and Wree, 1985]. Disturbances to the prefrontal cortex may be integral to the pathogenesis of hyperactivity and anxiety disorders. Anatomical and functional alterations in frontostriatal circuits have been implicated in the pathogenesis of attention-deficit/hyperactivity disorder [Solanto, 2002]. The dorsolateral prefrontal, lateral and parietal regions are heteromodal association cortices in which information from lower-order sensory systems is integrated into higher-order percepts and functions. These regions together are thought to form a broadly distributed action-attention system that supports the maintenance of attention focus and successful inhibitory control of unwanted impulses [Mesulam, 1998; Peterson et al., 2000].

Brain injury in humans [Bechara et al., 2000; Weingarten, 1999] including pediatric traumatic brain injury [Vasa et al., 2004] as well as other animal models of brain injury [Gonzalez et al., 2000; Lacroix et al., 2000; Rangel et al., 2003; Shah and Treit, 2003] support a role for the prefrontal cortex in the pathogenesis of anxiety disorders. We found that brain-injured animals exhibited anxiolysis early after injury that later resolved. In experimental studies, anxiolysis, apparent after lesions of the medial prefrontal cortex [Gonzalez et al., 2000; Lacroix et al., 2000; Rangel et al., 2003; Shah and Treit, 2003], is dependent upon post-lesion time [Rangel et al., 2003]. It has been suggested that injury to the medial prefrontal cortex ‘shocks’ the amygdala circuit, resulting in anxiolysis [Rangel et al., 2003]. Further studies are needed to better understand the circuitry that modulates these transient measures of anxiety after traumatic brain injury.
**Cognitive Function and Traumatic Brain Injury**

Deficits in spatial learning have been observed in children following traumatic brain injury [Lehnung et al., 2001]. Similarly, cognitive deficits have been reported after traumatic injury to the developing rodent brain [Adelson et al., 1997, 2000; Giza et al., 2005; Hicks et al., 2000; Prins and Hovda, 1998]. Adelson et al. [1997] investigated spatial learning and memory retention after ultrasevere and severe diffuse traumatic brain injury at postnatal day 17. Deficits in both spatial learning and memory retention were evident as early as 1 month after ultrasevere injury but were not apparent within the severely injured group. Prins and Hovda [1998] compared cognitive performance within the first several weeks after a fluid percussion brain injury at different stages of brain maturation. Although cognitive impairment was seen in animals that had been subjected to injury at postnatal day 21 and in the adult, no deficits were noted in animals that had been injured at postnatal day 17. Together, these findings suggest that both injury severity as well as age of the animal at the time of the injury may be determinants of cognitive function after traumatic brain injury. Importantly, neither of these studies addressed long-term cognitive function in those brain-injured animals that initially showed no cognitive deficits. In the present study, we offer the first evidence that cognitive decline after brain injury is delayed in onset. Whereas no cognitive deficits were noted in the juvenile cohort, injured animals in the adult cohort exhibited longer latencies in locating the hidden platform relative to either age-matched sham controls or juvenile brain-injured animals. This cognitive decline may in part reflect the progressive pattern of cell loss in the CA3 of the hippocampus. We found that neuronal density was significantly reduced in CA3 of adult brain-injured animals as compared with juvenile brain-injured animals. A similar association between cognitive deficits and neuronal loss in CA3 has been reported after traumatic injury to the adult rodent brain [Fox et al., 1998; Pierce et al., 1998]. Interestingly, others have reported cognitive deficits in young brain-injured animals without significant loss of neurons in the hippocampus [Giza et al., 2005]. Thus, loss of neurons in the hippocampus may not necessarily be a prerequisite for cognitive impairment.

The CA3 region of the dorsal hippocampus has been implicated in spatial learning [Kesner et al., 2000]. This subfield is important during the acquisition phase of memory [Kesner et al., 2000; Lassalle et al., 2000]. Temporary inactivation or lesions (25%) of the dorsal hippocampus cause impairments in both acquisition and retrieval of spatial memory [Moser et al., 1993; Moser and Moser, 1998; Riedel et al., 1999]. Selective neurotoxic lesions of the CA3 region likewise cause spatial learning and memory impairments [Stubley-Weatherly et al., 1996; Sutherland et al., 1983]. In this study, impairments in acquisition but intact memory retention in the probe trials were revealed. It is conceivable that impairments in memory retention in the brain-injured animals might be revealed using additional probe trials earlier during hidden water maze training.

In contrast to the water maze, brain-injured mice did not show impairments in recognizing the novel location of a familiar object. Although both tests require hippocampal function, it is possible that they involve different neural circuitry. As novel location recognition and spatial learning in the hidden water maze both require the use of visual extra maze cues, it is unlikely that potential loss of visual cortical regions following brain injury contributed to the acquisition deficits in the hidden water maze. In addition, there was no difference in the ability of the brain-injured and sham control mice in locating the visible platform location. In this task, mice were placed into the water in nine different locations around the pool circumference and the starting location was changed for each trial. Brain-injured mice would be expected to show impairments in locating the visible platform compared with sham controls if they had severe damage in visual cortical regions.

**Progressive Pathogenesis after Traumatic Brain Injury**

Traumatic brain injury resulted in prolonged pathogenesis in cortical and subcortical brain regions. This was evidenced by expansion of the cortical lesion and progressive neuronal loss in the CA3 region of the hippocampus and dorsal thalamus. A similar extended period of pathogenesis has likewise been reported in the cortex [Chen et al., 2003; Fox et al., 1998; Pierce et al., 1998; Smith et al., 1997], hippocampus and laterodorsal thalamus [Baldwin et al., 1997; Carbonell and Grady, 1999; Pierce et al., 1998] after traumatic injury to the adult brain. The lack of effects of traumatic brain injury on sensorimotor function suggests remarkable plasticity in the injured sensorimotor cortex.

The early neuronal loss in cortex and hippocampus has been attributed to direct mechanical damage, excitotoxicity, acute inflammatory response and oxidative damage which are rapidly initiated after traumatic brain injury [Faden et al., 1989; Woodroofe et al., 1991]. However, long-term pathophysiology has received limited attention. Putative pathophysiological mechanisms include apoptosis, ongoing inflammation and trauma-induced...
neurodegeneration [Conti et al., 1998; Fox et al., 1998; Grady et al., 2003; Raghupathi et al., 1998; Smith et al., 1997]. Apoptotic cell death has been reported in both the cortex and hippocampus after traumatic brain injury [Conti et al., 1998]. Neuronal apoptosis in the cortex occurs over a period of several months, whereas apoptosis peaks in the CA3 of the hippocampus at 48 h after injury [Conti et al., 1998]. Ongoing inflammation could also promote progressive cell death [Grady et al., 2003; Smith et al., 1997]. For example, reactive astrocytes are apparent in the cortex and hippocampus up to 1 year after injury [Smith et al., 1997]. Finally, delayed cell death in the thalamus, a hallmark of traumatic injury to the cortex of the adult [Conti et al., 1998] and developing brain [Tong et al., 2002], has been attributed to degeneration of thalamocortical fibers [De Bilbao et al., 2000].

Although the regional pattern of neuronal vulnerability is similar in the adult and developing brain after traumatic injury, the impact on brain structure and function is likely to be different. The extended period of pathogenesis is occurring during brain maturation. Thus, how it may affect ongoing developmental processes including synaptogenesis, gliogenesis, myelination and neurogenesis remains unclear. Here, we show a significant reduction in the number of immature neurons (doublecortin positive) in the dentate of the adult cohort relative toagematched sham controls. In contrast, no differences were noted in the juvenile cohort relative to controls. These findings suggest a fundamental change in how immature neurons may be generated in the hippocampus and emphasize the need for further study into how prolonged pathogenesis after traumatic brain injury influences both developmental processes and behavior.

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References


Traumatic Injury to the Immature Brain


