Repetitive mild traumatic brain injury induces ventriculomegaly and cortical thinning in juvenile rats

Corey Goddeyne,1,2 Joshua Nichols,1,2 Chen Wu,1 and Trent Anderson1
1University of Arizona, College of Medicine-Phoenix, Phoenix, Arizona; and 2School of Life Sciences, Arizona State University, Tempe, Arizona

Submitted 2 December 2014; accepted in final form 13 February 2015

TRAUMATIC BRAIN INJURY (TBI) is a significant health concern that affects more than 1.5 million Americans each year (Faul et al. 2010; Langlois et al. 2006; Rutland-Brown et al. 2006). At present, no single classification system has been developed that encompasses the host of clinical, pathological, behavioral, and cellular changes that occur as a result of TBI. In general, TBI is categorized into mild, moderate, and severe. Mild TBI (mTBI), including concussions, accounts for nearly 75% of all TBI cases (Barlow et al. 2004; Elder and Cristian 2009; Langlois et al. 2005, 2006; Míñolu et al. 2006). mTBI is often called an “invisible wound,” as it results in a minimal loss of consciousness (<30 min) and minimal acute neuropathological findings (Carroll et al. 2004; Morey et al. 2013; Smith et al. 2013). Consequently, mTBI is often difficult to detect and diagnose in the early acute stages after injury and may result in the incidence being underreported. After mTBI patients may experience cognitive and behavioral impairments including confusion, memory and attention deficits, and headaches (Barkhoudarian et al. 2011). These symptoms usually resolve completely within 2–3 wk after a single mTBI (Lovell et al. 2003; McCrea et al. 2003). However, especially with repeat injuries, these symptoms may persist for extended periods of time (Arciniegas et al. 2005; Halstead et al. 2010; Pellman et al. 2003).

Repetitive mTBI (rmTBI) significantly increases symptom severity (Collins et al. 2002), leads to longer-term cognitive and motor deficits (De Beaumont et al. 2007; Guskiewicz et al. 2011; Omale et al. 2010b), and increases the risk for developing dementia (Guskiewicz et al. 2005) and neurodegenerative disorders (Masel and DeWitt 2010; McKee et al. 2009, 2010; Plassman et al. 2000). Even a single mTBI event places patients at a greater risk for further TBI events and the ensuing consequences of rmTBI (Barkhoudarian et al. 2011; MacGregor et al. 2011; Tremblay et al. 2013; Zemper 2003). In contrast to a single mTBI event, rmTBI induces significant long-term structural changes to the brain including brain atrophy and enlargement of the ventricles (Giza 2006; Huh et al. 2007; Maxwell 2012; Smith et al. 2013; Wang et al. 2014). Currently no effective treatments are available to prevent the adverse complications associated with rmTBI. Development of new therapeutic strategies is contingent on an improved understanding of the underlying pathophysiological processes induced by rmTBI.

Recent public and research attention has focused on understanding rmTBI that occurs in adult athletes and military personnel. However, recent reports indicate that children may be particularly susceptible and sensitive to the effects of TBI (Barlow et al. 2010; Eisenberg et al. 2013; Field et al. 2003; Guskiewicz et al. 2000; Kontos et al. 2013). In children, TBI remains a leading cause of death and disability (Faul et al. 2010), with >10% experiencing a mTBI by the age of 10 (Barlow et al. 2010; Bruns and Hauser 2003). As with adults, the source of TBI varies greatly in children, but it may occur from a combination of events including accidents, abuse (shaken baby syndrome), or adolescent sport concussions. The pediatric brain is different from the adult brain owing to a host of ongoing neurodevelopmental processes including cortical hypertrophy, synaptogenesis, use-dependent pruning, enhanced glucose metabolism, increased neurotrophic factors, and altered excitatory amino acid receptors (Adelson 1999; Chugani et al. 1987; Friedman et al. 1991; Giza 2006; Insel et al. 1990). These processes have often been thought to confer children with an advantage in coping with brain injury, but...
repeated once daily for 5 days. Control animals were given anesthesia only. Postinjury day (PID) indicates number of days after the 5th rmTBI injury.

Fig. 1. Experimental timeline. Overview of the timeline used to model repetitive mild traumatic brain injury (rmTBI). Arrowheads represent time of single impact.

A tightly stretched Kimwipe secured to a Plexiglas stage (Fig. 2). The force of the impact caused the animal to break through the stretched Kimwipe, rotate 180°, and land dorsally on the foam pad below. The rat falls away from the impact weight, and no secondary impacts were observed. The animal’s movement after impact is not mechanically constrained, allowing simulation of the rotational and linear acceleration and deceleration forces most often associated with this type of injury. The animal was then placed in a supine position and monitored for righting reflex time. Righting reflex was defined as the animal’s ability to right itself from a supine to a prone position. Once righted and ambulatory the animal was placed back into its home cage and monitored daily. In the rmTBI animal group, this procedure was repeated once per day for a total of five impacts. Age-matched sham-injured animals were given anesthesia, underwent mock impacts, and were placed in a supine position to test righting reflex times. After the fifth sham injury or rmTBI, animals were again monitored daily but left for 14 days to recover before further experimentation. All “postinjury day” (PID) descriptions were calculated as days between last impact and day of death.

Brain fixation and tissue processing. At 14 days after injury (PID 14) the animals were deeply anesthetized with isoflurane and perfused transcardially with cold 0.9% saline followed by a fixative containing 4% paraformaldehyde. The brains were then removed and fixed in paraformaldehyde overnight. The following day brains were cryoprotected in two stages: 15% sucrose for 24 h followed by 30% sucrose for 24 h. Brains selected for MRI were then washed in PBS for 48 h. For immunofluorescence, 30-μm-thick sections were cut serially with a cryostat (Leica Biosystems) and stored at −20°C. Sections were then stained with the mature neuronal marker NeuN (Abcam, Cambridge, MA). In brief, the sections were first washed in PBS (2 × 15 min) before being permeabilized with 0.3% Triton X for 1 h (Abcam). Nonspecific binding was blocked with CAS-Block (Life Technologies). Finally, the sections were moved into the primary NeuN antibody diluted to 1:2,000 and incubated overnight on an orbital shaker at 4°C. The sections were then washed repeatedly in PBS and incubated with a Cy3 secondary antibody (1:1,000, Jackson Immunoresearch) in the dark at 4°C overnight. Images were taken of both control and rmTBI animals with an epifluorescent or confocal microscope.

Magnetic resonance imaging. MRI was performed on PID 14 brains that had been previously perfusion fixed. Imaging was performed on a Bruker Biospec 7.0-T small-animal MR scanner (Bruker Medizintechnik, Karlsruhe, Germany) with a 72-mm transmitter coil and a rat brain surface receiver coil. 3D RARE sequence was used to acquire coronal T2-weighted image (TE: 60 ms, TR: 3,000 ms, RARE factor: 8, resolution: 0.1 mm × 0.1 mm × 0.1 mm, matrix: 192 × 192 × 192, FOV: 19.2 mm × 19.2 mm × 19.2 mm, total acquisition time: 3 h 50 min) covering the posterior cerebellum to the frontal lobe. MRI data were analyzed with ImageI software.

Electrophysiological slice preparation. Coronal brain slices were made as described previously (Anderson et al. 2005, 2010; Iremonger et al. 2006). In brief, male Sprague-Dawley rats aged 38–45 days (PID 14–21) were deeply sedated via isoflurane inhalation and de-
capitated. Brains were quickly removed and placed in ice-cold (4°C) carboxygenated (95% O2-5% CO2) high-sucrose solution composed of (in mM) 126 NaCl, 26 NaHCO3, 2.5 KCl, 10 glucose, 1.25 NaH2PO4·H2O, 1 MgSO4·7H2O, 0.5 CaCl2·2H2O. The tissue was kept in this solution while 350-μm-thick coronal slices were taken with a vibratome (VT 1200; Leica, Nussloch, Germany). Brain slices were harvested from beneath the site of impact in rmTBI animals or from the corresponding area in sham-injured animals. Slices were incubated for 1 h in a water bath-warmed (32°C) container filled with carboxygenated artificial cerebrospinal fluid (aCSF) composed of (in mM) 126 NaCl, 26 NaHCO3, 2.5 KCl, 10 glucose, 1.25 NaH2PO4·H2O, 1 MgSO4·7H2O, 2 CaCl2·2H2O, pH 7.4. After the 1-h incubation, the slices were returned to room temperature before the tissue was moved to a recording chamber for whole cell patch-clamp recording.

**Whole cell patch-clamp recording.** Coronal brain slices prepared from rmTBI and sham-injured animals were placed in the recording chamber and immersed in carboxygenated aCSF maintained at a temperature of 32°C. Initial visualization and identification of cortical layers was done under ×4 brightfield magnification. Recordings were made from layer II/III neurons of motor cortex within the impact zone for rmTBI animals or the corresponding region in sham-injured animals. An upright microscope (Axioexaminer, Carl Zeiss) equipped with infrared differential interference contrast optics was used to acquire whole cell patch-clamp recordings from regular-spiking (RS) cortical pyramidal neurons. Current-clamp firing behavior was used to identify RS pyramidal neurons as previously described (Connors et al. 2010; Sun et al. 2006) to facilitate detection of inhibitory events.

**Data analysis.** Data were analyzed with pCLAMP (Axon Instruments), Prism (GraphPad), ImageJ (National Institutes of Health), and Mini Analysis (Synaptosoft) software and are presented as means ± SE. For immunohistochemical analysis of NeuN staining a region of interest (ROI) of the motor cortex was created with ImageJ software, and NeuN-positive cells within the ROI were manually counted. Cell count and density values are presented as the average cell count for three serial sections from each animal normalized to the width or area of the ROI, respectively. Electrophysiologically recorded spontaneous synaptic events were detected as previously described with automated threshold detection and manual verification (Nichols et al. 2015). R_in was calculated from the voltage response to the input of a current step (1 s, 50 µA). The adaptation index was calculated based on the ratio of the last interspike interval (F_last) divided by the second (F_2) as per the equation 100 × (1 − F_last/F_2). Pyramidal neurons often displayed a highly variable first interspike interval, and consequently F_2 was chosen for analysis. Firing frequency was calculated as the number of action potentials induced by a 1-s, 250-pA current step. Rheobase current was determined as the minimum current step (50 ms duration) that produced an action potential. Action potential threshold was calculated as the voltage at the maximum slope of the rheobase voltage recording (Nichols et al. 2015). Statistical significance was determined with an unpaired t-test, one-way ANOVA, or Kolmogorov-Smirnov test.
RESULTS

rmTBI is effectively modeled by repetitive weight drop. To model rmTBI in pediatric patients, we modified the weight-drop method recently published by Kane et al. (2012) for use with juvenile rats (Fig. 2A). Animals subjected to rmTBI demonstrated no gross morphological changes, identifiable surface deformations, or tissue loss at the site of the impact (Fig. 2B). The rmTBI procedure resulted in no incidence of scalp lacerations, and no immediate or late seizures were observed. As previously reported, the incidence of skull fractures or intracranial bleeding was low, and any animals displaying either where removed from further study (Kane et al. 2012). At PID 14–21 rat brains were removed for further experimentation. Acute slices prepared from rmTBI brains revealed marked structural changes including cortical thinning and ventriculomegaly (Fig. 2B). To determine the reproducibility of the rmTBI method, we tested the consistency of the impact force across 20 trials. A force meter (Chatillon DFM-10, Ametek Instruments) was placed at the base of the guide tube, and the peak impact force was measured across 20 trials. We found the average impact force with a 92-g weight to be highly consistent across trials with an average force of 7.890 ± 0.06 N and a maximum variation of <1 N (Fig. 2C, top).

In humans, the duration of loss of consciousness (LOC) is an important criterion in assessing the severity of a brain injury. While brain injury may occur in the absence of LOC, it is generally accepted that “mild” TBIs induce LOC between a few seconds and <30 min (Carroll et al. 2004; Smith et al. 2013). Assessing LOC in rats is difficult, but it has been indirectly evaluated by measuring the righting reflex time as an indicator of neurological restoration (Kane et al. 2012; Zecharia et al. 2012). Righting reflex time was measured after each sham or rmTBI impact as the time for an animal to recover from the supine to the prone position. Compared with sham-injured animals the righting reflex time was significantly increased across all 5 days (Fig. 2C, bottom). However, this increase in righting reflex time was not exacerbated by repeat sham (day 1 86.92 ± 8.8 s vs. day 5 88.59 ± 6.9 s) or rmTBI (day 1 200.60 ± 18.2 s vs. day 5 199.30 ± 14.6 s) injuries (P > 0.05 for both). Averaged across all five impact trials the righting reflex time remained significantly increased between in rmTBI (193.1 ± 6.7 s) vs. sham-injured (92.31 ± 4.0 s) animals.

MRI of rmTBI reveals significant ventriculomegaly and cortical thinning. To better assess the anatomical and structural changes to the brain following rmTBI, we performed T2-weighted MRI. Brains were perfusion fixed on PID 14 and ex vivo MRI imaging performed on control (n = 4) or rmTBI (n = 3) brains (Fig. 3). MRI imaging was performed from the frontal cortex to posterior cerebellum. To determine changes in cortical thinning, we measured the depth of the motor, somatosensory, and insular cortex across three regions—one region outside (bregma +2.3) and two regions within (bregma −0.6 and −3.5) the direct impact zone (Fig. 4A). The rmTBI was delivered by a 9-mm impact rod that spanned the region between bregma and lambda sutures in the rat. As imaging was performed ex vivo, we utilized anatomical landmarks to approximate the image location relative to the impact zone and published stereotaxic coordinates (i.e., bregma +2.3 mm, −0.6 mm, or −3.5 mm, respectively) (Paxinos and Watson 2007). In this way, we assessed changes in cortical depth across brain regions in the anterior-posterior as well as medial-lateral directions in both control and rmTBI animals. Substantial cortical thinning was observed in the motor cortex in all three brain regions, with up to a 46% decrease in cortical depth within the impact zone (Fig. 4B). Similarly, the depth of the somatosensory cortex was significantly reduced by over 25%, but this reduction was restricted to directly within the impact zone (i.e., bregma −0.6 mm and bregma −3.5 mm). Measurement of the depth of the insular cortex revealed no significant difference across all three brain regions examined (P > 0.05). We next...
performed similar measurements on the area of the third and lateral ventricles. While no significant difference in the area of the third ventricle was observed ($P > 0.05$), the lateral ventricle area increased up to 970% after rmTBI (Fig. 5). Within the impact zone (bregma $-0.6$ and $-3.5$), the lateral ventricle maximally increased from $1.37 \pm 0.2$ mm$^2$ to $13.30 \pm 1.0$ mm$^2$ ($P < 0.0001$). Outside of the direct impact zone (bregma $+2.3$), the lateral ventricles were again significantly increased from $0.84 \pm 0.1$ mm$^2$ to $4.40 \pm 0.4$ mm$^2$ ($P < 0.0001$). Collectively, the data reveal that rmTBI induces rapid and significant reduction in the depth of the cortex and ventriculomegaly that is most substantial at the site of impact.

**rmTBI induces no change in neuronal density or gross tissue damage.** To determine whether rmTBI altered the total number or density of neurons within the cortex, we performed immunohistochemical analysis with the neuron-specific marker NeuN (Fig. 6A). As the amount of rmTBI-induced cortical thinning was most pronounced in the motor cortex, we focused the analysis on this region. The decrease in cortical thickness resulted in an overall decrease in total NeuN-positive cells between sham-injured (476.1 ± 24) and rmTBI (296.1 ± 24) animals ($P < 0.0001$). However, analysis of the density of neurons (i.e., total neurons/area) revealed no significant change between sham-injured and rmTBI animals ($P = 0.21$) (Fig. 6B). Therefore, the data suggest that rmTBI induces a significant reduction in the volume of the cortex, but the cortex that remains is of similar neuronal density as that in sham-injured animals.

**rmTBI does not significantly alter electrophysiological properties of layer II/III motor neurons.** Structurally, this study has revealed that rmTBI induces a significant reduction in the depth of the cortex that is most widespread and profound within the region of the motor cortex. To determine whether these structural changes result in functional changes to the intrinsic and synaptic properties of neurons within the motor cortex we performed electrophysiological experiments. Specifically, we recorded from layer II/III motor cortex pyramidal neurons within the injury zone of rmTBI animals or from the corresponding area in age-matched sham-injured animals.

**Intrinsic excitability.** Intrinsic excitability refers to the propensity of a neuron to fire an action potential and is governed by the membrane properties, currents, and channels expressed by a neuron. Alterations to intrinsic excitability have been shown in numerous models of CNS disorders (Willmore 1990; Yang et al. 2007) and may contribute to the pathophysiology of rmTBI. To examine for changes in intrinsic excitability induced by rmTBI, we recorded under current clamp the response of sham-injured ($n = 10$) or rmTBI ($n = 14$) neurons to a series of hyperpolarizing and depolarizing steps ($-100$ pA to $350$ pA, $50$-pA steps). Analysis revealed no statistical difference in $R_i$ ($P = 0.38$), resting membrane potential ($P = 0.77$), or accommodation index ($P = 0.82$) between sham-
injured and rmTBI neurons (Fig. 7). Using a rheobase protocol (50 ms, 5-pA steps), we performed a more detailed analysis of action potential properties but again found no statistical difference in rheobase current ($P_{/H11005} = 0.73$), action potential threshold ($P_{/H11005} = 0.52$), or amplitude ($P_{/H11005} = 0.31$) (Fig. 8).

**Spontaneous activity.** The frequency of activity and strength of synaptic connections between neurons are fundamental to the way the brain processes and relays information. To investigate whether rmTBI disrupts or alters cortical synaptic excitability we again recorded from layer II/III pyramidal neurons in the motor cortex of sham-injured or rmTBI animals. First, under voltage clamp ($V_{\text{hold}} = 70$ mV), we examined for rmTBI-induced changes to spontaneous excitatory postsynaptic currents (sEPSCs). To minimize detection of inhibitory events, neurons were held near and positive of the $E_{\text{Cl}}$ ($V_{\text{hold}} = 70$ mV, calculated $E_{\text{Cl}} = -80$ mV) and only inward synaptic events were detected. Pharmacological isolation of glutamatergic events was avoided, as the resultant synaptic disinhibition may mask rmTBI-induced changes to network excitability. In neurons from rmTBI animals, there were no significant

---

**Fig. 6.** Effect of rmTBI on NeuN staining. A: representative epifluorescence and confocal images taken from sham injury ($n = 8$) or rmTBI ($n = 6$) stained with the neuron-specific marker NeuN (green). Scale bars, 1 mm. Images are at ×2.5, ×10, and ×20 magnification. B: bar graphs of average neuronal number (top) and density (bottom) within the motor cortex. Cell counts were made of NeuN-positive cells within standardized regions of interest (yellow dashed boxes in A). Note the substantial reduction of NeuN-positive cells after rmTBI but absence of neuronal density changes. ***$P < 0.0001$.**

**Fig. 7.** Intrinsic membrane properties are not altered by rmTBI. A: representative current-clamp recordings in response to intracellular current steps ($-100$ pA to $350$ pA, 1 s) in layer II/III pyramidal neurons from sham-injured ($n = 10$) or rmTBI ($n = 14$) animals. Note the similarity in the intrinsic cellular response. B: average intrinsic membrane properties. No significant difference was found for input resistance ($P = 0.38$) or resting membrane potential ($P = 0.77$). C: comparison of firing properties of a sham-injured and an rmTBI animal. Left: plot of average firing frequency vs. current ($f-I$ curve). Right: adaptation index [first interevent interval ($IE_{\text{first}}$) between action potentials/last interevent interval ($IE_{\text{last}}$)].
changes in the average interevent interval ($P = 0.77$), amplitude ($P = 0.94$), decay time ($P = 0.82$), or charge transfer ($P = 0.34$) of sEPSCs (Fig. 9). Next, we similarly examined for changes in spontaneous inhibitory postsynaptic currents (sIPSCs). Inhibitory events were pharmacologically isolated with bath application of the glutamate receptor antagonist kynurenate (2 mM). To enhance detection fidelity of inhibitory synaptic events, a modified high-intracellular Cl$^-$/H$^+$ internal solution was used as previously described (Anderson et al. 2010; Sun et al. 2006). Again, no significant change was observed in sIPSC properties including interevent interval ($P = 0.90$), amplitude ($P = 0.74$), decay time ($P = 0.33$), and charge transfer ($P = 0.46$). Representative traces and summary of these results are shown in Fig. 10.

Finally, the effects of rmTBI in humans are often subtle and may not be reflected in changes to baseline synaptic activity but only become evident during periods of high activity or demand. The observed reduction of cortical depth and neuronal number in rmTBI animals relative to sham-injured animals may result in loss of peak network or synaptic activity. To test these possibilities we challenged pyramidal neurons from sham-injured ($n = 15$) or rmTBI ($n = 18$) animals with the convulsant 4-aminopyridine (4-AP, 100 μM). Bath application of 4-AP for 15 min induced a rapid decrease in interevent interval of sEPSCs recorded in neurons from both sham-injured (53.59 ± 5.6 ms) and rmTBI (81.21 ± 20.0 ms) animals (Fig. 11A). The amplitude of sEPSCs was similarly increased by 4-AP in neurons from both sham-injured
However, neither the interevent interval nor amplitude during application of 4-AP was statistically different between neurons recorded from sham-injured and rmTBI animals ($P > 0.05$). Overall, this suggests that despite a significant loss of the depth of the motor cortex in rmTBI animals, the injury fails to alter excitatory or inhibitory synaptic properties or the potential peak state of synaptic excitability.

Fig. 10. Inhibitory spontaneous synaptic activity is not altered by rmTBI. A: voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) in sham-injured ($n = 15$) or rmTBI ($n = 18$) animals. B: overlay of sham-injured and rmTBI scaled average sIPSC. C: average sIPSC IEI and amplitude for sham and rmTBI. No significant difference was determined for IEI ($P = 0.90$) or amplitude ($P = 0.74$). D: average sIPSC kinetic properties. No significant difference was detected between sham injury and rmTBI for sEPSC decay time ($P = 0.33$) or charge transfer ($P = 0.46$). $V_{\text{hold}} = -70 \text{ mV}$.

Fig. 11. rmTBI does not enhance the response to the convulsant 4-aminopyridine (4-AP). A: voltage-clamp recordings of sEPSCs from sham-injured ($n = 14$) or rmTBI ($n = 12$) animals during bath application of 4-AP (100 $\mu$M). B and C: bar chart and cumulative probability curves of sham injury, sham injury during 4-AP, and rmTBI during 4-AP for IEI (B) or amplitude (C). Bath application of 4-AP induced a significant decrease in IEI and amplitude of sEPSCs. However, the effects of 4-AP on sEPSC IEI and amplitude were not statistically different between sham injury and rmTBI. $V_{\text{hold}} = -70 \text{ mV}$. *$P < 0.05$, **$P < 0.01$. 

$3275$
DISCUSSION

In the pediatric population, TBI remains a significant health concern that is known to place patients at risk for adverse long-term cognitive and behavioral changes. TBI may vary in severity, but >75% of all TBI is classified as mild (Cassidy et al. 2004; Elder and Cristian 2009; Langlois et al. 2005; Minino et al. 2006). In this study, we sought to determine how rmTBI affects the pediatric brain. To effectively model human rmTBI, we modified a recently developed method for inducing rmTBI in adult animals (Kane et al. 2012) for use in juveniles. This rmTBI weight-drop method produced highly consistent impact forces across trials. The impacts occurred in a nonrestrained animal and have been shown to effectively model the direct, acceleration, and deceleration forces determined to be important to human TBI (Gennarelli and Thibault 1982; Holbourn 1943; Kane et al. 2012; Ommaya et al. 1967; Panzer et al. 2014). After mTBI, animals exhibited a significant increase in righting reflex time that suggests a brief injury-induced period of sensory and/or motor dysfunction. In contrast to what has generally been reported after single mTBI (Mychasiuk et al. 2014) or rmTBI in adult animals (Kane et al. 2012), rmTBI in juvenile animals induced significant structural changes to the brain including cortical atrophy and ventriculomegaly. This is supported by recent evidence that indicates that children may be more prone to the effects of repeat concussions (Eisenberg et al. 2013; Field et al. 2003). Neuronal specific immunostaining revealed that the cortical atrophy was accompanied by a loss of total cortical neurons. However, this overall neuronal loss was not due to a specific reduction in cortical density. The cortical atrophy was most pronounced in the motor cortex, with up to a 46% decrease in cortical thickness beneath the site of injury in rmTBI animals. At PID 14 the significant structural changes to the motor cortex were not accompanied by significant changes in the intrinsic or synaptic properties of layer II/III pyramidal neurons at rest or under convulsant challenge. Overall, our results indicate the effectiveness of this new weight-drop method for reliably inducing a clinically relevant rmTBI. The select changes induced by rmTBI in juvenile rats suggest a potentially unique pathophysiological response to TBI in children.

Modeling repetitive mild traumatic brain injury. Recent attention by patients, families, researchers, and the media has highlighted the significant short- and long-term consequences of rmTBI (Creeley et al. 2004; Longhi et al. 2005; Shitaka et al. 2011). Critical to understanding the pathophysiological mechanisms that drive rmTBI has been the development of new, clinically relevant models. Effective modeling of rmTBI requires an induced injury that reflects the type of impact and forces known to occur in mTBI and that results in neuropathological and clinically relevant outcomes. mTBI is characterized as occurring in a closed skull with minimal skull fractures and minimal tissue loss after a single mTBI. The impact of the mTBI induces direct force to the skull that translates into acceleration, deceleration, and shearing forces in the brain that are thought to be important to the injury process (Duhaime et al. 2012). Several models of TBI exist, including controlled cortical impact and fluid percussion (Xiong et al. 2013), but these require a craniotomy and/or a fixed skull that inadequately models these forces. Limited data exist on the exact biomechanical forces that would be classified as “mild” or concussion inducing, but the most comprehensive data have been obtained from head impact telemetry devices placed within athletes’ helmets. An in-depth review of the combined telemetry impact studies revealed that concussion is correlated with g-forces above 100g (Beckwith et al. 2013). In our study, calculated impact forces were on average 26.8g and well within the “mild” range [i.e., g-force = (F = ma)/9.8 m/s²; F = 7.89 N, m = 30g (P20-25 rat)]. The method used in this study overcomes these limitations and effectively models both the biomechanical forces of the impact and has been shown to induce clinically relevant cognitive and behavioral changes (Gennarelli and Thibault 1982; Kane et al. 2012; Meaney and Smith 2011; Mychasiuk et al. 2014; Panzer et al. 2014).

Repetitive mild traumatic brain injury induces significant neuropathology. A single mTBI often resolves quickly and has generally not been associated with any significant neuroimaging abnormalities (Belanger et al. 2007; Petchprapai and Winkelmann 2007; Morey et al. 2013). As a result, mTBI is often referred to as an “invisible wound” and is difficult to diagnose. Whether a single mTBI induces long-term deficits is currently a source of significant debate (Carroll et al. 2004; Klein et al. 1996; Konrad et al. 2011; Vanderploeg et al. 2005; Vasterling et al. 2012; Yuh et al. 2014). It is clear, however, that when a patient receives multiple mTBIs within a short period of time it results in more severe symptoms, a longer recovery period, and increased risk for serious long-term consequences (Guskiewicz et al. 2000, 2003). In contrast to a single mTBI event, rmTBI patients show clear neuropathological findings including enlarged ventricles (ventriculomegaly) and cortical atrophy (Huh et al. 2007; Smith et al. 2013). These findings are supported by the results of this study, which indicate that after rmTBI the lateral ventricles may be increased up to 970% while the thickness of the cortex may be reduced by up to 46%. The interplay and timing of the enlarged ventricles and cortical atrophy remain to be determined. However, the cortex appears not to be simply compressed by the enlarged ventricles, as no change in cortical neuronal density was observed. These changes were not observed after rmTBI in adult animals (Kane et al. 2012), suggesting a potentially unique response to TBI in juvenile animals. While the impact force used in this study was “mild,” the neuropathological findings in rmTBI animals are more significant and may highlight the deleterious effects of receiving multiple mTBIs.

In humans, rmTBI can induce a neurodegenerative disease termed chronic traumatic encephalopathy (CTE) (Gavett et al. 2011; Smith et al. 2013) that has been most commonly found in professional athletes (McKee et al. 2009; Omalu et al. 2010a, 2010b) or soldiers exposed to blast or concussive injury (Goldstein et al. 2012). CTE can currently only be diagnosed on autopsy but results in degeneration of brain tissue (i.e., cortical atrophy) and ventriculomegaly similar to what was observed in this study. Additional characteristics of CTE include tau accumulation, cognitive impairments, memory loss, confusion, and depression (McKee et al. 2009, 2010; Miller 1966). Further work examining these characteristics will be required to determine whether the neuropathological outcome of rmTBI in this study is indicative of underlying CTE.

Cortical excitability is not altered early after repetitive mild traumatic brain injury. Structurally, this study revealed extensive thinning of the cortex that was most pronounced beneath the site of injury in the motor cortex. Immunohistochemical
staining revealed that rmTBI reduced the total number of cortical neurons, but this was not accompanied by a decrease in neuronal density. The significant loss of motor cortex is supported by several studies that have indicated persistent motor dysfunction and abnormalities in the motor cortex after mTBI (De Beaumont et al. 2011, 2012; Tremblay et al. 2014). In addition, many of the behavioral deficits associated with rmTBI such as balance, reaction time, and visual memory involve high levels of integration across cortical regions (Co-vassin et al. 2008; Khurana and Kaye 2012; Slobounov et al. 2007) that are thought to be governed by input and output from layer II/III cortex (Douglas and Martin 2004; Kamper et al. 2013). This agrees with a recent study that found mTBI induces specific dendritic degeneration and synaptic reduction in cortical layer II/III pyramidal neurons (Gao and Chen 2011). As such, in this study we began by examining for rmTBI-induced changes in the intrinsic and synaptic properties of layer II/III pyramidal neurons within the motor cortex.

A neuron’s intrinsic excitability determines the probability it will fire an action potential, and the output pattern of that firing has been shown to contribute to the pathophysiology of several other neurological disorders (Bush et al. 1999; Prince and Connors 1986; Prinz et al. 2013; van Zundert et al. 2012). However, at PID 14 we investigated several possible measures of intrinsic excitability and found no significant differences between our rmTBI and sham-injured groups. This finding is supported by recent work from our lab where even severe TBI in juvenile rats failed to alter the intrinsic properties of cortical pyramidal neurons (Nichols et al. 2015). At a synaptic level, again at PID 14 no significant changes were found in the strength, frequency, or kinetics of either excitatory or inhibitory synaptic neurotransmission following rmTBI. To our knowledge this is the first study to investigate detailed intracellular electrophysiological changes following rmTBI.

In humans, the use of transcranial magnetic stimulation from 72 h to 2 mo after mTBI has shown increases in intracortical inhibition (Miller et al. 2014). Young athletes who have sustained multiple concussions have also been reported to have abnormal intracortical inhibition (De Beaumont et al. 2007, 2011; Tremblay et al. 2011). While no change in inhibition onto pyramidal neurons was observed in this study, future examination of the impact of rmTBI directly on other cortical layers and inhibitory interneurons may reveal distinct changes. Given the significant neuropathological changes following rmTBI, it is surprising to find no accompanying electrophysiological changes. The lack of synaptic excitability changes observed after rmTBI in this study contrast with recent findings after severe TBI from our lab in juvenile rats (Nichols et al. 2015) and from previous reports in adult animals (Cantu et al. 2014). As this study only examined animals at 14 days after injury it will be important to examine changes that may occur in the acute and more chronic time points after rmTBI. The data suggest that juvenile rats have a unique injury phenotype after rmTBI that may be in part due to high levels of plasticity in the juvenile brain (Akbik et al. 2013; Grutzendler et al. 2002; Li and Asante 2011; Selemon 2013), ongoing development (Kolb and Gibb 2011), and/or potential trauma-induced postnatal neurogenesis (Gregg et al. 2001; Kolb et al. 2007).

The effects of mTBI may often be subtle and only evident when the cortex is challenged with a high-demand task (Abdel et al. 2009). With the clear loss of mature neurons and significant cortical atrophy, we hypothesized that rmTBI animals may have a reduced upper limit of synaptic activity that would be evident only when the cortex was put under “stress.” To test this, we examined the synaptic properties of sham-injured and rmTBI animals during application of 4-AP, a potassium channel blocker and known convulsant. 4-AP has been shown to increase synaptic excitability (Boudkkazi et al. 2011; Buckle and Haas 1982) and to affect cortical pyramidal neuron intrinsic excitability (Higgs and Spain 2011; Shu et al. 2007). As expected, both the frequency and amplitude of spontaneous excitatory activity were increased from control periods by bath application of 4-AP. However, the effects of 4-AP were not statistically different between sham-injured and rmTBI animals. Therefore, even when cortical excitability is pharmacologically increased, rmTBI animals remain equally responsive and able to enhance synaptic activity compared with sham-injured animals. However, in this study we only tested the response of a saturating dose of 4-AP that produces a near-maximal level of synaptic activity. The use of a dose-response protocol may reveal subtler changes in network excitability or 4-AP sensitivity after rmTBI.

In conclusion, rmTBI has been associated with serious clinical consequences including chronic traumatic encephalopathy and an increased risk for the development of dementia and neurodegenerative diseases (McKee et al. 2009, 2010; Omalu et al. 2010a, 2010b). In this study, we found that rmTBI can be effectively modeled in young animals with a modified weight-drop method. The impacts can be consistently delivered and replicate clinically relevant impact forces and structural changes including cortical atrophy and ventriculomegaly. This method of inducing mTBI has also recently been shown in juvenile (Mychasiuk et al. 2014) and adult (Kane et al. 2012) animals to induce clinically relevant changes to cognition and behavior. At present, the findings from this study suggest that the pathophysiology of rmTBI may be unique when occurring in pediatric patients. An improved understanding of how the pediatric brain responds to rmTBI may help identify novel therapeutic targets, influence pediatric treatment, and improve “return to game” decision making in adolescents.

GRANTS
Support for this project was provided by the University of Arizona College of Medicine-Phoenix.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: C.G., J.N., and C.W. performed experiments; C.G., J.N., C.W., and T.A. interpreted results of experiments; C.G., J.N., C.W., and T.A. prepared figures; C.G. and T.A. drafted manuscript; C.G., J.N., C.W., and T.A. edited and revised manuscript; C.G., C.W., and T.A. approved final version of manuscript; T.A. conception and design of research.

REFERENCES


J Neurophysiol • doi:10.1152/jn.00970.2014 • www.jn.org


