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Traumatic Brain Injury During Development Reduces Minimal Clonic Seizure Thresholds At Maturity

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Abstract

Post-traumatic seizures affect 12 – 35% of children after traumatic brain injury (TBI) and are associated with worse cognitive and functional outcome, even after adjustment for severity of injury. Unfortunately, experimental models of pediatric post-traumatic epilepsy are lacking, and pathogenesis remains poorly understood. We have applied a standard model of TBI in immature rats to determine the effect of TBI on electroconvulsive seizure thresholds later in life.

Male rats underwent controlled cortical impact to left parietal cortex on post-natal day (PND) 16-18. Hindbrain, forebrain, and limbic seizure thresholds were assessed, respectively, by tonic hindlimb extension (THE), minimal clonic, and partial psychomotor seizure responses during adolescence (PND 34 - 40) and at maturity (PND 60 - 63). Post-traumatic seizure thresholds were compared to those obtained in age- and litter-matched sham craniotomy and naïve controls.

TBI during immaturity had no clear effect on THE seizure thresholds. In contrast, TBI lowered minimal clonic seizure thresholds at maturity ($p < 0.05$ vs. sham or naïve rats), but not during adolescence. Consequently, minimal clonic seizure thresholds increased with age for sham and naïve rats but remained similar for TBI rats during adolescence and at maturity. TBI also tended to lower partial psychomotor seizure thresholds, which were determined only during adolescence ($p < 0.1$ vs. naïve).

Controlled cortical impact causes both focal cortical injury at the site of impact and ipsilateral hippocampal neuronal death. Since minimal clonic seizures are mediated by the forebrain, partial psychomotor seizures by the limbic system, and THE seizures by the brainstem, the observed pattern of changes in post-traumatic seizure thresholds is not surprising. The apparent age-dependent effects of TBI, however, are unexpected and likely due to a combination of attenuated maturational increases

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and progressive epileptogenesis. Additional study is needed to delineate the relative contributions of these processes. Given the sustained reduction in post-traumatic minimal clonic seizure thresholds, controlled cortical impact may hold promise as an experimental model of pediatric post-traumatic epilepsy.

Keywords

head injury; controlled cortical impact; seizure threshold; developing rat; rodent

Introduction

Traumatic brain injury (TBI) is a leading cause of childhood mortality and morbidity (Langlois, et al., 2004). Post-traumatic seizures affect 12 - 35% of children suffering moderate or severe TBI and are associated with worse cognitive and functional outcome, even after adjustment for severity of injury (Chiaretti, et al., 2002; Hahn, et al., 1988; Keenan, et al., 2004; Ong, et al., 1996; Ratan, et al., 1999). Typically, pediatric post-traumatic seizures occur during the initial week post-TBI; however, post-traumatic epilepsy, which is defined as recurrent, unprovoked seizures occurring later than one-week post-TBI, develops in 10 - 20% of children after severe TBI (Appleton and Demellweek, 2002; Barlow, et al., 2000). Suppressive therapy for pediatric post-traumatic epilepsy is often ineffective; nearly 60% of cases are intractable to medical management (Appleton and Demellweek, 2002; Barlow, et al., 2000). Further, although anticonvulsant medications are frequently given to children to suppress seizure activity during the first week post-TBI, prophylactic anticonvulsant therapy does not reduce the incidence of post-traumatic epilepsy or improve outcomes (Adelson, et al., 2003).

Experimental investigations of pediatric post-traumatic seizures and post-traumatic epilepsy are sparse, and the pathogenesis of either remains poorly understood. One factor that contributes to our limited understanding is a lack of clinically relevant experimental models (Pitkanen and McIntosh, 2006; White, 2002). As an initial step toward developing a clinically relevant model of pediatric post-traumatic epilepsy, we have applied a standard model of TBI in immature rats and determined the effect of TBI during development on electroconvulsive seizure thresholds later in life. We hypothesized that TBI during immaturity would result in lower seizure thresholds during adolescence and at maturity.

Methods

Male Sprague-Dawley rats were obtained from Charles Rivers Laboratories (Raleigh, NC) on post-natal day (PND) 7 - 10, with the day of birth considered PND 0. Rats were housed in litters of 10 with a lactating female until weaning on PND 21 - 23. After weaning, rats were housed 3 - 5 per cage and allowed free access to food and water. All cages were kept in a temperature- and light-controlled (12 h on/12 h off) environment. All experimental procedures were approved by The University of Utah Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Animals. All surgical procedures were performed using aseptic technique.

Our study includes three experimental groups (n = 20/group): TBI, sham craniotomy, and naïve. Rats were randomized to experimental group on PND 16 - 18. In order to control for maternal rearing characteristics, randomization was distributed evenly within litters. Rats underwent TBI or sham craniotomy on PND 16 - 18 and seizure testing on PND 34 - 40 and PND 60 - 63. MRI assessment of cerebral injury was performed, *ex vivo*, after euthanasia on PND 80 - 85.

Traumatic Brain Injury

On PND 16 - 18, rats undergoing TBI were anesthetized with 3% isoflurane for induction followed by 2 - 2.5% isoflurane for the duration of surgical preparation. Core temperature was monitored via a rectal probe and controlled at $37 \pm 0.5^\circ\text{C}$. The rat was placed into a stereotaxic frame (David Kopf, Tujunga, CA) and a craniotomy ($6 \times 6\text{-mm}$) was performed over the left parietal cortex (centered around the point 4-mm anterior and 4-mm lateral to bregma) using a high-speed dental drill. The dura and bone flap were left in place until immediately before TBI. A temperature probe (2.28-mm outside diameter, Physiotemp Corp., Clifton, NJ) was placed through a burr hole into the left frontal lobe, and brain temperature was controlled at $37 \pm 0.5^\circ\text{C}$. Once the craniotomy was complete, anesthesia was reduced to 1% isoflurane for a 5-min equilibration period. TBI was then performed by controlled cortical impact (Pittsburgh Precision Instruments, Pittsburgh, PA) to left parietal cortex (5-mm rounded tip, 4 m/sec velocity, 2-mm deformation, and 100 msec duration) (Dixon, et al., 1991). Immediately after TBI, isoflurane was increased to 2 - 2.5%, and the bone flap was replaced and secured with cold-curing acrylic. The scalp incision was sutured closed and washed with providone-iodine solution. Triple antibiotic ointment and bupivacaine 0.25% were applied topically to provide local antibiotic and analgesic therapy. Isoflurane was stopped, and rats were allowed to recover in a temperature-controlled chamber. Once fully awake, rats were returned to their dams and littermates. Sham rats underwent identical surgical craniotomy, equilibration, and closure procedures without TBI. Naïve rats underwent randomization with no further intervention.

Seizure Testing

Three standard electroconvulsive seizure paradigms were applied via transcorneal stimulation to assess hindbrain, forebrain, and limbic seizure thresholds during adolescence (PND 34 - 40) and at maturity (PND 60 -63) (Otto, et al., 2004). All seizure testing was performed during daylight hours. All experimental groups were tested concurrently. Tetracaine (0.5%) eye drops were used to provide corneal anesthesia. Each rat was tested only once each day. Testing for a single seizure paradigm was completed during a single day, and testing for different seizure paradigms was separated by at least 48 hours of rest (Freeman and Jarvis, 1981).

Tonic hindlimb extension (THE) seizures, which are mediated by the brainstem, were used to assess hindbrain seizure thresholds (Browning, 1985; Browning, 1986; Browning, et al., 1981; Eells, et al., 2004). THE seizures begin as tonic forelimb extension, spread to hindlimb flexion, and terminate in full tonic hindlimb extension (180 degrees to the torso). Minimal clonic seizures, which are mediated by the forebrain, were used to determine forebrain seizure thresholds (Browning, 1985; Browning, 1986; Eells, et al., 2004). Minimal clonic seizures are manifest as rhythmic face and forelimb clonus, rearing and falling, and ventral neck flexion. Partial psychomotor seizures, which are mediated by the limbic system, were used to assess limbic seizure thresholds (Barton, et al., 2001). Rhythmic face movements, forelimb clonus, dorsal neck flexion, rearing and falling, and/or transient gait wobbliness or ataxia characterize partial psychomotor seizures.

A stimulator previously described (Woodbury and Davenport, 1952) (frequency of 60 Hz, sinusoidal current pulse of 0.2 msec, and stepwise variation of current intensity) was used to evaluate THE or minimal clonic seizure responses on PND34 and 60 or PND 37 and 63, respectively. A Grass stimulator (Model S4B, frequency of 6 Hz, stimulus duration of 3 sec, 0.2 msec rectangular current pulse, and stepwise variation of current intensity) was used for partial psychomotor seizure testing on PND 40 (Barton, et al., 2001). Due to difficulty applying the necessary 3-sec transcorneal stimulus in larger rats, partial psychomotor seizure testing was not performed at maturity. Population seizure thresholds were determined for each seizure paradigm via the staircase estimation procedure (Finney, 1971). Briefly, the stimulation intensity for each rat was determined by the response of the prior rat (presence or absence of

seizure). The current stimulus was varied across 4 - 7 intensities for each seizure paradigm, with several rats being tested at each stimulus. From these data, full convulsive current (CC) curves were generated and CC₁₋₉₉ values were calculated using Probit analysis in the statistical program Minitab (State College, PA). Seizure thresholds were defined, *a priori*, as the CC at which 50% of rats are expected to exhibit seizures (CC₅₀).

Lesion Assessment by MRI

On PND 80 - 85, rats were anesthetized with 4 - 5% isoflurane and perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hr, then stored in sterile, isotonic phosphate-buffered saline at 4° C. MRI was performed *ex vivo* using a gradient echo sequence (MP rage, inversion time 1700 msec, flip angle 12 degrees, echo time 12 msec, echo spacing 26.7 msec, and 5 averages) on a 1.5 Tesla scanner (Siemens Corporation, New York, NY).

MRI images were analyzed using ANALYZE 7.0 software (Mayo Clinic, Rochester, MN). The MRI scan data were used for two purposes: (1) to provide gross anatomical inspection of the brain to qualitatively demonstrate lesion size and location (and to verify no obvious structural damage in the sham and naïve conditions) and (2) to quantitatively estimate total lesion volume, which typically overestimates histologically verified volume by about 25% (Nishi, et al., 2007; Onyszchuk, et al., 2007). Analysis techniques were adapted from methods originally developed to determine lesion and total brain volumes in human TBI subjects (Hopkins, et al., 1997; Wilde, et al., 2005). Briefly, essentially following the methods outlined by Schweinhardt et al. (Schweinhardt, et al., 2003), all brains from subjects in the naïve group were co-registered, and the images were smoothed to create a uniform average brain of the control, non-operated naïve subjects that was, in turn, used to create a single average brain template. One problem with *ex vivo* MRI of the brain that has been removed from the cranium is that all skull, vascular, and meningeal fiduciary landmarks have been removed, including where to define the original surface of the now damaged cerebral cortex. Developing an average template of the control brain permitted comparison of each individual TBI brain and enabled lesion volume estimation. Each TBI brain was overlaid on the average template by aligning the inter-hemispheric fissure and base of the brain, since the cortical impact lesions were on the dorsal surface. Utilizing the ANALYZE® thresholding technique, common parenchymal signal between the TBI brain and the average brain template was identified. Lesion volume was defined as the difference between total brain parenchymal volume of the TBI brain and that of the average brain template. This technique identifies total hemispheric volume loss relative to the average brain template, which includes but is not necessarily restricted to the lesion cavity. We chose this method due to morphological artifact associated with *ex vivo* MRI scanning. Brains were fixed prior to imaging and held in solution without bony support, which created some tissue distortion and thresholding challenges and caused inaccuracies in more direct calculations of absolute lesion volume. However, application of this analysis technique to the post-mortem MRI provided a means of directly visualizing three-dimensional lesion characteristics, estimating lesion size, and comparing gross brain morphology between TBI, sham, and naïve rats.

Statistical Analysis

CC curves and seizure thresholds were compared by Probit analysis. Anesthetic duration and rectal and brain temperatures were compared by 2-tailed t-test. Rat weights were compared by analysis of variance for repeated measures, with Tukey's test for post-hoc analysis. Probit analysis was performed using the statistical program MINITAB (State College, PA). All other statistical testing was performed using SPSS 11.0 for MAC (Chicago, IL). A *p*-value of < 0.05 was considered significant.

Results

Anesthetic duration (32 ± 9 min for TBI rats and 27 ± 5 min for sham rats) was similar between groups. Similarly, rectal and brain temperatures (37.3 ± 0.26 and 37.2 ± 0.17 °C for TBI rats and 37.2 ± 0.25 and 37.1 ± 0.15 for sham rats) did not differ between groups. Overall survival for the study was 80% for TBI rats, 85% for sham rats, and 95% for naïve rats. One TBI rat died after controlled cortical impact, 6 rats died during adolescence (1 TBI rat after THE seizure testing and 2 TBI and 3 sham rats after limbic seizure testing), and 1 rat died as an adult (a naïve rat after minimal clonic seizure testing). All experimental groups demonstrated adequate weight gain during the experimental period. However, TBI rats displayed less robust weight gain than naïve rats ($p < 0.05$, Fig. 1). Weight gain for sham rats was similar to either TBI or naïve rats.

Electroconvulsive seizure thresholds were higher for THE than minimal clonic seizures in all experimental groups ($p < 0.05$). This was expected because higher current intensities are needed to excite hindbrain vs. forebrain structures via transcorneal stimulation (Browning, 1985). THE seizure thresholds increased with age for all experimental groups ($p < 0.05$, Table 1). THE seizure thresholds were similar for TBI, sham, and naïve rats during adolescence. At maturity, THE seizure thresholds did not differ between TBI and sham rats; however, thresholds were lower for either TBI or sham rats compared to naïve rats ($p < 0.05$).

Minimal clonic seizure thresholds increased with age for sham and naïve rats ($p < 0.05$), but not for TBI rats (Table 1). Consequently, TBI rats had similar minimal clonic seizure thresholds during adolescence and at maturity. During adolescence, minimal clonic seizure thresholds did not differ between experimental groups (Table 1). At maturity, minimal clonic seizure thresholds were lower for TBI rats than either sham or naïve rats ($p < 0.05$). Minimal clonic seizure thresholds did not differ between sham and naïve rats at maturity.

Psychomotor seizure thresholds, which were determined only during adolescence, did not differ between experimental groups (Table 1). There was a trend toward lower psychomotor seizure thresholds for TBI compared to naïve rats, however, this failed to reach statistical significance ($p = 0.085$).

Representative coronal sections, taken through the dorsal hippocampus, and three-dimensional cortical surface reconstructions from MRI scans depict the location and extent of cerebral injury (Fig. 2). The majority of TBI rats had cavitory lesions; however three showed ipsilateral hypertrophy. All TBI rats had evidence of cortical surface damage, although it was less evident among those rats with hypertrophy on coronal sections. The reported lesion volume includes hippocampal loss ipsilateral to the contusion. The MRI techniques used do not permit specific quantitative assessment of the degree of hippocampal injury; however, qualitative evaluation suggests some variability in the extent hippocampal involvement. No sham or naïve rat had evidence of cavitory or hippocampal damage on MRI scanning. The mean ($\pm$ SD) lesion volume after TBI was $379 (\pm 133)$ mm³. When normalized to total brain volume (to account for variability in individual brain size), this corresponded to $30 (\pm 14)\%$ of total brain volume.

Discussion

Controlled cortical impact to the left parietal cortex in immature rats lowers minimal clonic seizure thresholds at maturity. Partial psychomotor seizure thresholds during adolescence tend to be lower after TBI; however, statistical significance was not reached. In contrast, controlled cortical impact had no clear effect on THE seizure thresholds; both sham and TBI rats showed reduced THE thresholds at maturity compared to naïve rats. Minimal clonic seizures are mediated by the forebrain, partial psychomotor seizures are mediated by the limbic system, and THE seizures are mediated by the brainstem (Browning, 1985; Browning, 1986; Eells, et

al., 2004). Since controlled cortical impact causes not only focal cortical injury at the site of impact but also ipsilateral hippocampal neuronal death (Hall, et al., 2005; Tong, et al., 2002), this pattern of post-traumatic seizure threshold changes is not surprising. However, the observed reduction in THE seizure thresholds for both TBI and sham rats is unexpected and raises concern that anesthetic administration during immaturity may alter seizure susceptibility. Exploration of this concern is intriguing, but requires additional investigation and is beyond the scope of the current study.

Interestingly, TBI lowers minimal clonic seizure thresholds at maturity but not during adolescence. The reasons for this age-dependence are not clear but likely include attenuation of maturational increases in seizure thresholds and/or progression of epileptogenesis. Although electroconvulsive seizure thresholds typically increase with age, in TBI rats minimal clonic seizure thresholds remained nearly identical during adolescence and at maturity. This suggests that TBI attenuates normal maturational increases in seizure thresholds. Alternatively, the process of post-traumatic epileptogenesis may be too gradual for detection by electroconvulsive seizure threshold testing during adolescence. In our study, adolescent seizure testing was conducted only 2-weeks post-TBI, whereas adult seizure testing was done 6 weeks post-TBI. In adolescent and mature rats subjected to TBI, latency periods of 2 weeks to 7 months are reported between TBI and seizure onset (D'Ambrosio, et al., 2004; Kharatishvili, et al., 2006). Similarly, in children the median interval between severe TBI and seizure onset is 2.9 years (range: 8 months to 5 years) (Appleton and Demellweek, 2002). Likely, the observed age-dependent effects of TBI on minimal clonic seizure thresholds are due to a combination of attenuation of maturational changes and progressive epileptogenic changes. Further study is necessary to better elucidate the interaction between these two processes.

Prior experimental investigations of the effects of TBI on electroconvulsive seizure thresholds are lacking; however, TBI-induced neuronal excitability is substantiated using other experimental methods. Susceptibility for either kainic acid or pentylenetetrazole induced seizures is increased after TBI in adult rats (Golarai, et al., 2001; Zanier, et al., 2003). Spontaneous generalized tonic clonic seizures are reported immediately after TBI in adult rats and adenosine A1_A receptor knock-out mice (Kochanek, et al., 2006; Nilsson, et al., 1994). Post-traumatic epilepsy is reported after TBI induced by fluid percussion injury in both adolescent and adult rats (D'Ambrosio, et al., 2004; D'Ambrosio, et al., 2005; Kharatishvili, et al., 2006). The mechanisms underlying neuronal excitability, increased seizure susceptibility, and spontaneous seizures post-TBI have not been well defined, but may include the acute release of excitatory amino acids, altered NMDA receptor expression, inhibitory interneuron loss, mossy fiber sprouting, and/or gliosis (D'Ambrosio, et al., 2004; Giza, et al., 2006; Nilsson, et al., 1994; Statler, 2006). In adult rats, post-traumatic dentate gyrus hyperexcitability is specifically associated with mossy fiber sprouting and inhibitory interneuron loss (Golarai, et al., 2001; Statler, 2006; Toth, et al., 1997). Similar mechanistic studies are needed in immature rats.

Nutritional differences may affect cerebral maturation and seizure susceptibility. Although weight gain during the experimental period was less robust in TBI rats (vs. naïve), our findings are not likely explained by nutritional deficits. The observed post-traumatic alterations in electroconvulsive seizure thresholds were not generalized, but specific to those brain regions that were most severely affected by TBI. Additionally, weight gain was reduced but still adequate among TBI rats.

Our study includes limited assessment of histopathological damage, which is an acknowledged limitation. Lesion volume was assessed by *ex vivo* MRI using comparison to an average naïve brain template. This method detects differences that include, but are not strictly limited to, the lesion. Additionally, a subset of TBI rats unexpectedly showed hypertrophy ipsilateral to TBI

on coronal sections taken through the dorsal hippocampus. Given that all rats underwent identical TBI, with strict temperature control during the peri-injury and recovery periods, it is unclear why a subset of rats displayed a different response to injury. Maternal rearing characteristics are influential to rat pup development and may alter response to stress in immature rats (Pryce and Feldon, 2003; Smotherman, 1983). Although maternal rearing may have contributed to injury response in our study, those rats showing post-injury hypertrophy were not reared in a single litter.

Possible mechanisms for post-injury hypertrophy include increased growth factor secretion, altered maturational pruning, compensatory sprouting, and enhanced neurogenesis. In models of seizure-related cerebral injury in immature rats, relative resistance to cell loss (vs. mature rats), aberrant mossy fiber sprouting in the absence of cell loss, and functional deficits out of proportion to the degree of histological tissue injury are reported (Ben-Ari and Holmes, 2006). Additionally, delayed neurogenesis, which is associated with greater spontaneous seizure activity, is seen months after status epilepticus is induced by lithium-pilocarpine in immature rats (Cha, et al., 2004).

In our study, individual seizure testing responses were used to generate population seizure thresholds for THE, minimal clonic, and partial psychomotor seizures after TBI. Although we have shown that TBI in immature rats lowers minimal clonic seizure thresholds at maturity and may lower partial psychomotor seizure thresholds during adolescence, the experimental techniques used do not allow correlation between the extent or distribution of cerebral injury and individual seizure susceptibility. Thus, further investigation, including detailed confirmatory histological analysis of the extent and patterns of post-traumatic cerebral injury and correlation between lesion size and post-traumatic seizure susceptibility, is needed in our model.

Electroconvulsive seizure testing is most commonly used for preclinical assessment of anticonvulsant medication efficacy. Strain- or mutation-related enhanced seizure susceptibility correlates with lower electroconvulsive seizure thresholds in mice (Loscher, et al., 1986; Otto, et al., 2004); however, limited investigation has been done to correlate changes in electroconvulsive seizure thresholds with the incidence of spontaneous seizures after a cerebral insult. Our study documents decreased electroconvulsive seizure thresholds for minimal clonic seizures after TBI during immaturity. These data are strongly supportive, but not conclusive, evidence for an increased risk for spontaneous seizures after TBI during immaturity. The controlled cortical impact may hold promise as an experimental model of pediatric post-traumatic epilepsy; however, further investigation, including documentation of spontaneous post-traumatic seizures, is necessary.

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Figure 1.

Weight gain during the experimental period for TBI (filled circles and solid line), sham (filled triangles and dashed line) and naïve (filled squares and dotted line) rats. Although all groups demonstrate adequate weight gain during the experimental period, TBI rats have less robust weight gain than naïve rats ($p < 0.05$, analysis of variance for repeated measures). Weight gain for sham rats is similar to either TBI or naïve rats.

Figure 2.

Representative MRI findings two months post-TBI are depicted by coronal sections taken through the dorsal hippocampus and three-dimensional cortical surface reconstructions. TBI was delivered to the left parietal cortex on PND 17. MRI scans were obtained *ex vivo* after euthanasia on PND 80 - 85. **(A.)** Coronal sections taken through the dorsal hippocampus depict the range of focal destructive lesions induced by TBI. Comparison sections are shown for both sham and naïve rats. Most TBI rats had cavitary lesions; however a few showed ipsilateral cortical and hippocampal hypertrophy, even though the same procedure was used to induce TBI in all cases. Subject 228-02 has the smallest destructive lesion, and subject 228-03 has the largest. Subject 225-05 shows ipsilateral hypertrophy. **(B.)** Three-dimensional surface renderings of the MRI findings depict the extensive cortical damage present after TBI. Comparison renderings are shown for sham and naïve rats. Cortical surface damage was evident in all TBI rats, including those with smaller focal destructive lesions (e.g. subject 228-02) or ipsilateral hypertrophy (e.g. subject 225-05) on the coronal images.

Table 1

Seizure Thresholds (CC₅₀ Values in mamps)

	Adolescence (PND 34 - 40)			Maturity (PND 60 - 63)			% Change with Age		
	TBI	sham	naïve	TBI	sham	naïve	TBI	sham	naïve
THE	26.9 (23.7, 29.6)	30.4 (28, 33)	27.8 (25.1, 30.5)	33.7 [†] (32.1, 35.3)	34.5 [†] (33.0, 35.8)	36.5 [*] (35.1, 37.8)	25	13	31
Minimal Clonic	11.6 (9.3, 13.8)	12.9 (10.9, 14.8)	12.2 (10.0, 14.1)	12 [‡] (10.5, 13.5)	14.8 [*] (13.6, 16)	14.7 [*] (13.4, 15.9)	3	15	20
Psychomotor	87.2 [#] (85.4, 88.9)	87.8 (86.4, 89.2)	89.1 (87.6, 90.5)	--	--	--	--	--	--

Data are presented as median (IQR).

- * $p < 0.05$ vs. adolescence for respective experimental group;
- ϕ $p < 0.05$ vs. sham of same age;
- $\dagger$ $p < 0.05$ vs. naïve of same age;
- $\#$ $p < 0.1$ vs. naïve of same age