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Repetitive Brain Injury of Juvenile Mice Impairs Environmental Enrichment-Induced Modulation of REM Sleep in Adulthood

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Abstract

Traumatic brain injuries (TBI) are a common and costly ongoing public health concern. Injuries that occur during childhood development can have particularly profound and long-lasting effects. One common consequence and potential mediator of negative outcomes of TBI is sleep disruption which occurs in a substantial proportion of TBI patients. These individuals report greater incidences of insomnia and sleep fragmentation combined with a greater overall sleep requirement meaning that many patients are chronically sleep-deprived. We sought to develop an animal model of developmental TBI-induced sleep dysfunction. Specifically, we tested the hypothesis that early (postnatal day 21), repeated closed head injuries in Swiss-Webster mice, would impair basal and homeostatic sleep responses in adulthood. Further, we asked whether environmental enrichment, a manipulation that improves functional recovery following TBI and has been shown to alter sleep physiology, would prevent TBI-induced sleep dysfunction and alter sleep-modulatory peptide expression. In contrast to our hypothesis, the mild, repeated head injury that we used did not significantly alter basal or homeostatic sleep responses in mice housed in standard laboratory conditions. Sham-injured mice housed in enriched environments exhibited enhanced REM sleep and expression of the REM-promoting peptide pro-melanin concentrating hormone, an effect that was not apparent in TBI mice housed in enriched environments. Thus, TBI blocked the REM-enhancing effects of environmental enrichment. This work has important implications for the management and rehabilitation of the TBI patient population.

Keywords

mild traumatic brain injury; REM sleep; non-REM sleep; environmental enrichment; orexin; neurodegeneration

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Introduction

Traumatic brain injury (TBI) represents an ongoing and critical public health issue. Every year, approximately 1.7 million people in the United States sustain a TBI with almost 90% of those injuries being mild, which often go unreported and subsequently untreated (Coronado, McGuire et al., 2012; Vos, Alekseenko et al., 2012). Despite the characterization of these injuries as mild, it is becoming increasingly clear that there are significant and persistent consequences of this class of injuries. In particular, brain trauma suffered by children can produce lifelong consequences (Keenan and Bratton, 2006). Survivors of childhood injuries are more likely to suffer from a variety of neurological and neuropsychiatric conditions, substance abuse, cognitive deficits, legal and social issues and a large variety of other disease states as compared to uninjured cohorts (Stewart-Scott and Douglas, 1998; Jorge, Starkstein et al., 2005; Corrigan, Bogner et al., 2013; Corrigan, Cuthbert et al., 2014). The specific mechanisms that link childhood TBI to poorer outcomes are incompletely understood but likely include both direct neurological damage and impairment of ongoing neurodevelopmental activities.

One potential proximate mediator of these kinds of broad negative outcomes is disruption of sleep wake cycles. Sleep disturbance is an extremely common sequela of TBI with studies estimating that 30–70% of TBI patients experience sleep disturbance following their injuries (Viola-Saltzman and Musleh, 2016; Sandsmark, Elliott et al., 2017). Sleep problems can manifest as an initial period of hypersomnia that resolves into long-term insomnia and sleep fragmentation (Castriotta, Wilde et al., 2007; Billiard and Podesta, 2013). Further, in addition to sleep fragmentation, TBI survivors often require more sleep (up to 2 additional hours/24 hours; a phenomenon termed pleiosomnia) (Sommerauer, Valko et al., 2013). Importantly, this is not limited to patients with severe injuries as sleep disturbance is common to mild TBI (concussion) survivors particularly among those that have experienced repeated injuries (Ouellet, Savard et al., 2004; Bryan, 2013; Theadom, Cropley et al., 2015). Finally, there have been reports of the loss of arousal-promoting orexin/hypocretin neurons as well as other hypothalamic arousal-modulating cells (e.g. histamine and melanin-concentrating hormone) in TBI patients (Baumann, Bassetti et al., 2009; Valko, Gavrilov et al., 2015).

This combination of poor sleep and increased sleep demand has the potential to significantly impair neurological recovery from injury. Sleep dysfunction has serious consequences for normal neural physiology even in otherwise healthy brains (McEwen, 2006). Specifically, sleep disruption can result in exhaustion of central energy stores, prolonged neuroendocrine stress responses, the induction of inflammatory responses, and oxidative damage (Leproult, Copinschi et al., 1997; Kong, Shepel et al., 2002; Guzman-Marin, Suntsova et al., 2003). It is therefore not entirely surprising that sleep disruption is a negative predictor of recovery from TBI and patients with sleep problems report greater disability even after controlling for potential confounds like depression, anxiety, and pain (Mollayeva, Mollayeva et al., 2016; Mollayeva, Pratt et al., 2016). Moreover, sleep fragmentation produces significant neurobehavioral deficits including problems with memory, concentration and mood (Blunden, Lushington et al., 2005; Blunden, Lamond et al., 2005) in otherwise healthy

individuals. Thus, TBI survivors that already exhibit cognitive issues and nervous system dysfunction are likely to experience much greater negative consequences from sleep fragmentation.

Animal models of post-TBI sleep have been relatively variable (for a recent review see (Sandsmark, Elliott et al., 2017)). Depending on the specific type and severity of injury, some studies have reported acute increases in sleep, while others reported more chronic effects, and still others described changes in sleep consolidation all measured over different periods of time after injury (Sandsmark, Elliott et al., 2017). However, to the best of our knowledge, no study has yet investigated the effects of repeated mild head injuries in young animals with the specific goal of investigating adult sleep.

Prolonged housing in enriched environments can serve as an animal model for sustained cognitive and physical rehabilitation. Housing animals in enriched environments (EE) can reduce secondary neurodegeneration and otherwise improve functional outcomes after brain injury (Bondi, Klitsch et al., 2014; Schreiber, Lin et al., 2014; Weil, Karelina et al., 2016). Further, EE can improve sleep efficiency and alter sleep architecture (Gutwein and Fishbein, 1980). What is not clear, however, is whether enrichment can improve sleep in the injured brain as there is evidence that both sleep disruption and brain injury can impair enrichment-induced plasticity (Mirmiran, Uylings et al., 1983; Giza, Griesbach et al., 2005; Sta Maria, Reger et al., 2017). Therefore, the goal of this study is to determine if multiple mild traumatic brain injuries in juvenile mice lead to disrupted sleep cycles in adulthood, and if this effect is prevented by an EE. Finally, we examined orexin/hypocretin and melanin-concentrating hormone expression in the lateral hypothalamus to determine whether changes in the expression of these sleep-modulating neuropeptides mediate the effects of injury and enrichment on sleep.

Experimental Procedures

Animals and experimental design

All animal procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and were conducted according to NIH guidelines. In all cases, animals were randomly assigned to groups and investigators were blinded to experimental conditions.

Swiss-Webster mice were obtained from Charles River Laboratories (Kingston, NY) and bred in our facilities. Mice were supplied *ad libitum* access to food (Harlan Teklad #7912) and filtered tap water, along with a cotton nestlet. At three weeks of age male pups were randomly assigned to an experimental group in a full factorial design (Experimental design Fig. 1), weaned (at 21–24 days of age), and then subjected to three mTBI (described below) or sham procedures over the course of one week. Following the final procedure, mice were left undisturbed (except for weekly cage changes and body mass measurements) in either standard or enriched housing conditions until approximately 9 weeks of age (i.e., adulthood). Standard housing consisted of group housing mice (2–5/cage) in standard rodent polypropylene cages (27.8 × 7.5 × 13 cm) with access to a cotton nestlet and normal *ad libitum* access to food and water. Mice assigned to enriched conditions were group housed

(3–8/cage) in a large rat-sized cage (45×25×20cm) with plastic enrichment items (tubes, huts, blocks) and a running wheel as well as several cotton nestlets and *ad libitum* access to food and water. The animals were housed in mixed-condition groups with both sham- and injured mice in each cage. Littermates were split into different conditions to avoid any litter-wise effects. Enrichment items were changed twice/week to provide additional variety. All animals were bred and housed in 14:10 light:dark cycles with lights off at 14:00 EST. Mice were weaned immediately after their first mild TBI or sham procedure. Total n=41 with n=9–11/group.

Repeated mild traumatic brain injury—A repeated mild, closed head, traumatic brain injury was induced using an Impact One device (Leica Biosystems, Richmond IL). Mice (21-day-old) were anesthetized with isoflurane (3% induction, 1.5% maintenance), secured in a stereotaxic frame (Stoelting, Wood Dale, IL) and the skull was exposed using aseptic surgical technique. This is a modification of a procedure we have extensively optimized (Karelina, Sarac et al., 2016; Weil, Karelina et al., 2016; Karelina, Gaier et al., 2017; Karelina, Gaier et al., 2017). The impactor was placed gently on the surface of the skull (–1 mm AP, 1 mm ML relative to bregma) and then the stainless-steel impactor tip (round 2mm in diameter) was driven into the closed skull to a depth of 1 mm at 3 m/s (dwell time 30 msec). The procedure was repeated every other day for a total of three injuries. This treatment reliably produces mild traumatic brain injury without skull fracture, hemorrhage or mortality. The sham procedure was identical except the impactor was slowly lowered into contact with the skull and then retracted.

Transmitter implantation and EEG/EMG recording

Upon reaching adulthood, mice were deeply anesthetized under isoflurane vapors (3% induction, 1.5% maintenance) and implanted with PhysioTel F20-EET (Data Sciences International [DSI], St. Paul, MN, USA) biotelemetry units to allow acquisition of electroencephalogram and electromyogram potentials as described previously (Borniger, Gaudier-Diaz et al., 2015). Following immobilization in a stereotaxic apparatus, a midline incision was made extending between the posterior margin of the eyes and the midpoint of the scapulae. The skull was exposed and cleaned, and two stainless steel screws (00–96 × 1/16; Plastics One, Roanoke VA, USA) were inserted through the skull to make contact with the underlying dura mater. These screws served as cortical electrodes. One screw was placed 1 mm lateral to the sagittal suture and 1 mm anterior to bregma. The other screw was placed contralaterally 2 mm from the sagittal suture and 2 mm posterior to bregma. The transmitter was inserted into a subcutaneous pocket along the back of the animal. A set of leads was attached to the cortical electrodes and secured with dental cement. Another set of leads was inserted into the trapezius muscles for EMG measurement. The surgical procedures were performed using aseptic technique, and buprenorphine (0.05 mg/kg, SC) was administered to provide post-operative analgesia along with supplemental warmth (heating pad) until the animals were mobile. Ibuprofen was supplied in the drinking water for 3 days following surgery for additional analgesia. Following surgery, mice were singly housed and their cages were placed on top of receiver boards (RPC-1; DSI) in ventilated cabinets. These boards relay telemetered data to a data exchange matrix (DSI) and a computer running Ponemah

software (version 6.3; DSI, St. Paul, MN, USA). Mice were allowed to recover from the surgery for two weeks prior to beginning sleep recordings.

Sleep deprivation

A sleep deprivation protocol was performed to examine the homeostatic response to sleep deprivation following baseline sleep recordings. Total sleep deprivation was accomplished via ‘gentle disruption’ during the first six hours of the rest phase (ZT 0–6), followed by an 18h recovery period (as described in (Oyanedel, Kelemen et al., 2015)). If EEG/EMG biopotentials showed signs of sleep and the animal displayed a sleeping posture, the cage was gently tapped to arouse the mouse. If this did not prevent sleep, then the experimenter would briefly handle the mouse or disrupt its bedding. As this method is dependent on live monitoring of EEG/EMG and sleep posture, this method cannot eliminate all sleep, and short episodes (< 10 sec) of ‘micro-sleep’ persisted. The timeline for experimental procedures is summarized in Fig 1.

Immunohistochemistry and Silver Staining

After the 18 h recovery period following sleep deprivation, mice were injected intraperitoneally with sodium pentobarbital in saline (30%, 0.2 mL) and were transcardially perfused with ice-cold 0.1M PB followed by 4% paraformaldehyde-PB. Brains were dissected and post-fixed in 4% PFA-PB overnight, and then cryoprotected in 30% sucrose in 0.2M PB until sunk. Brains were then frozen and stored at -80°C until cryostat sectioning. 20 μm coronal sections were taken throughout the extent of the brain and serially collected into cryoprotectant or 0.1M PB. Free floating sections were stored at -20°C or 4°C (in 4% PFA-PB for silver staining) until downstream processing.

Immunostaining (Hypocretin-1/Orexin-A and melanin concentrating-hormone) was completed on free-floating 20 μm cryostat sections. Following 5×10 min washes in 1xPBS, sections were blocked (for 1 hour) in 2.5% normal donkey serum, 0.1% Triton-X100, and 1xPBS. Sections were then incubated for 24h at room temperature in primary antibody (1:400; Goat anti-Orexin-A #sc-8070 or 1:200; Goat anti-pro-melanin-concentrating hormone #sc-14509) in blocking solution. Sections were washed 3 times in PBS, then incubated in donkey anti-goat 2° Ab (1:200; AlexaFluor 488), washed again and then mounted onto slides with VectaShield + DAPI mounting media (Vector Labs). Sections were examined bilaterally at 10X magnification and cells expressing orexin/hypocretin and separately pro-MCH in the lateral hypothalamus were counted using ImageJ by an investigator blinded to the experimental conditions. The hypocretin/orexin and MCH positive cells were counted in serial sections spanning the lateral hypothalamus and normalized to the number of sections that contained the lateral hypothalamus.

Silver staining and axon damage assessment

Sections were subjected to a silver staining protocol according to the manufacturer’s instructions as previously described (Weil, Gaier et al., 2014) using an FD NeuroSilver Kit (FD Neurotechnologies) to examine the effects of repeated mTBI on axon degeneration as an index of overall brain damage. Tissue was scored qualitatively by an investigator blinded to the experimental conditions (0 = minimal staining, 3 = dark staining throughout white

matter tracts including the corpus callosum). The assigned score reflects global axon degeneration as assessed in representative tissue sections collected through the entire forebrain. White matter damage following TBI is evident in multiple tracts, including the corpus callosum, cingulum, external and internal capsule, anterior commissure, and optic tract.

Sleep architecture and spectral analyses

Raw biopotentials were analyzed in 10s epochs via the automated rodent sleep scoring module in Neuroscore (DSI) as previously described (Borniger, Gaudier-Diaz et al., 2015). Delta and theta ratio criteria, as well as EMG threshold values (for scoring of NREM, REM, and wake, respectively) were adjusted on a per-animal basis to ensure accurate scoring across the experimental set. Delta band was set at 0.5–4.0 Hz, and the theta band was set at 6.0–9.0 Hz. EEG signals were band pass filtered at 0.3–35 Hz and EMG signals were filtered at 10–100 Hz as previously described (Veasey, Valladares et al., 2000). Artifact detection thresholds were set at 0.4 mV for both EMG and EEG, and if >10% of an epoch fell outside this threshold, the entire epoch was scored as artifact. Wake was characterized by high frequency and low voltage EEG accompanied by high voltage EMG. NREM (i.e., slow wave sleep) sleep was characterized by low frequency and high voltage EEG (predominant delta), accompanied by low voltage EMG. REM (i.e., paradoxical) sleep was characterized by high frequency, low voltage EEG (predominant theta) and EMG values (Fig 2a shows representative traces). Epochs (10s) were collapsed into 2 h bins for subsequent graphing and statistical analyses. The percent time spent in each vigilance state was calculated across a recording period (light or dark phase), as well as the number of vigilance state transitions and duration of each bout in each vigilance state as a measure of sleep fragmentation. For spectral analyses, biopotentials were visually inspected, cleaned of artifacts, and subjected to Fast-Fourier Transforms (Hamming signal window; normalized to # of samples in spectrum). Periodogram data were collected in 10s epochs for an entire day of biopotential recording. Spectra were generated across a frequency range of 0.49–23.97 Hz (~0.5 Hz frequency window). For analysis of responses to sleep deprivation, spectra from the first 6 hours of recovery sleep were normalized to the same time of day on the previous day (when no sleep deprivation occurred).

Statistics

We screened for outliers within groups using the Grubb's test. One mouse was removed from sleep analysis due to poor EEG/EMG signal quality. ANOVAs were completed for analyses of sleep-wake data averaged across the day and night. Factorial ANOVAs were used to compare mean values (e.g., % NREM sleep during the light phase) with housing condition and injury condition as independent variables. If a significant ($p < 0.05$) F value was detected, post-hoc Tukey HSD tests were completed to conduct inter-group comparisons.

Results

Silver Staining

Silver staining revealed mTBI-induced axon damage that persisted at 9 weeks following injury, and did not differ as a function of housing condition (Fig 2a–c, summarized in d) (Sham/Standard mean silver score: 0.5 ± 0.547 , TBI/Standard: 1.5 ± 0.8366 , Sham/Enriched: 0.571 ± 0.789 , TBI/Enriched: 1.6 ± 0.5477). This indicates similar pathological responses in standard and enriched mice induced by juvenile injuries when assessed in adulthood.

Orexin/Hypocretin Staining.

There were no effects of either housing condition or injury status on orexin/hypocretin neuron counts in the lateral hypothalamus (Fig 2e–h, summarized in i).

Pro-Melanin Concentrating Hormone Staining.

There were no main effects of either injury ($F_{1,21}=0.27$, $p>0.05$) or enrichment ($F_{1,21}=3.63$, $p>0.05$) on the number of pro-MCH-immunoreactive cells in the lateral hypothalamus (**Fig j–m, summarized in o**). However, there was a significant interaction ($F_{1,21}=4.733$, $p=0.041$) that was mediated by greater numbers of immunoreactive cells in the sham-enriched compared to sham-standard groups ($p>0.05$). There were no other differences in pro-MCH expression.

Locomotor Activity

During the second day of baseline recordings, there was a main effect of enrichment on spontaneous locomotor activity (two-way ANOVA: $F_{1,29} = 5.018$, $p = 0.0329$), where mice that were housed in enrichment during adolescence moved more than those that were in standard housing conditions. There was no main effect of TBI nor an interaction ($p > 0.05$). Post-hoc testing revealed that the main effect was largely driven by the sham/enriched group, as they differed significantly from shams in standard housing (Tukey HSD Sham-Standard vs. Sham-Enriched: $q = 3.87$, $dF = 29$, $p = 0.0487$) (Fig 3).

Baseline Sleep

There was a main effect of housing on the percentage of time spent in REM sleep during the day (baseline 2-day average two-way ANOVA: $F_{1,28} = 9.677$, $p = 0.0043$; Fig 4b–c). Post-hoc testing revealed that sham/enriched mice increased their amount of REM sleep (Tukey HSD Post-hoc Sham/Standard vs. Sham/Enriched $q = 4.723$, $dF = 28$, $p = 0.012$). This effect of EE was not evident in mTBI mice (Tukey HSD Post-hoc: TBI/Standard vs. TBI/Enriched: $q = 1.6$, $dF = 28$, $p = 0.6737$). During the night, a similar phenotype was evident, with a main effect of housing on the percent of time spent in REM sleep (two-night baseline average two-way ANOVA: $F_{1,28} = 18.2$, $p = 0.0002$; Fig 4b–c) without a main effect of mTBI nor an interaction. Post-hoc testing revealed that sham/enriched mice increased nighttime REM sleep compared to their counterparts housed in standard conditions (sham/standard vs. sham/enriched Tukey HSD post-hoc $q = 5.099$, $dF = 28$, $p = 0.0062$). Mice that underwent mTBI exhibited an intermediate phenotype characterized by enhanced nighttime

REM sleep compared to sham/standard mice (sham/standard vs. TBI/enriched Tukey HSD: $q = 4.057$, $dF = 28$, $p = 0.0367$), but were not different from their standard-housed counterparts (TBI/standard vs. TBI/enriched Tukey HSD: $q = 3.489$, $dF = 28$, $p = 0.0875$). These data suggest that multiple early life mild TBIs impair the REM-enhancing effect of EE. Whole spectrum (0.5–25 Hz) analyses of REM sleep during baseline recording showed that enrichment enhanced REM θ (6–9Hz) power primarily in mice that received sham surgery only although this was not statistically significant ($p = 0.08$; Fig 4d).

There was no main effect of TBI or housing (or interaction) on the number of REM bouts during the day. However, during the night, housing condition influenced the number of REM sleep episodes, with enriched mice showing more REM sleep bouts during the night (main effect of housing: $F_{1,28} = 16.97$, $p = 0.0003$; main effect of TBI $F_{1,28} = .4711$, $p = 0.4981$; interaction $F_{1,28} = 2.218$, $p = 0.1476$; Fig 4e). Tukey post-hoc analyses revealed that sham-surgery animals that were reared in enriched conditions had significantly more nighttime REM bouts than their counterparts reared in standard conditions (Sham/Standard vs. Sham/Enriched $dF = 28$, $q = 5.795$, $p = 0.0017$). This effect was not evident among animals that received TBIs as juveniles (Standard/TBI vs. Enriched/TBI $dF = 28$, $q = 2.551$, $p = 0.2927$).

There was no main effect of housing or TBI treatment on NREM sleep (Fig 4f–g).

Sleep Deprivation and Recovery

The sleep deprivation procedure employed here significantly reduced REM and NREM sleep followed by compensatory sleep once sleep deprivation ended. However, there were no significant effects of either housing condition or injury revealed by this procedure. There was more REM sleep in the sham/enriched mice (greater percentage of time and numbers of bouts) after sleep deprivation but this was similar to before the injury. No differences in NREM spectral power were noted during recovery sleep, indicating that mice that received TBI and/or enrichment showed equivalent homeostatic responses to acute (6h) sleep deprivation.

Discussion

In this study, we sought to investigate whether a mild, but repeated, closed head injury experienced during development would impair sleep behavior in adulthood. Additionally, we sought to determine whether EE could prevent TBI-induced sleep disruption. Our injury paradigm produced a moderate amount of axonal degeneration without inducing large-scale neuropathology. However, in contrast to our predictions, repeated juvenile traumatic brain injuries did not significantly impair basal sleep physiology or the homeostatic response to sleep deprivation. Interestingly, housing animals in EE, a technique which has been shown to mitigate some of the deleterious consequences of TBI, altered REM sleep physiology but only in sham injured animals. Thus, traumatic brain injury blocked the REM sleep-modulatory effects of EE.

Long term sleep disruption is a common sequela of traumatic brain injuries and particularly those that occur early in development (Billiard and Podesta, 2013; Theadom, Cropley et al., 2015; Sandsmark, Elliott et al., 2017). However, the long-term effects of repeated mild TBI

on sleep physiology in adulthood remains unknown. Thus, we had hypothesized that our model of mild, repeated traumatic brain injury in juvenile mice would produce persistent alterations in sleep (timing, intensity, etc.) in adulthood. Here, we reported that sleep was essentially unaltered by juvenile TBI. This does not, however, rule out the possibility that transient disruptions in sleep occurred in the early post-injury period and resolved prior to our sleep assessments. That is consistent with reports that fluid percussion injuries transiently increase sleep time over the first six hours after injury but sleep changes resolve by five weeks after injury (Rowe, Harrison et al., 2014; Rowe, Striz et al., 2014).

Despite the lack of alterations in sleep physiology among standard-housed mice, EE did reveal a key TBI effect. Specifically, EE significantly altered REM sleep in sham, but not TBI, mice. Environmental enrichment is a common manipulation that consists of increasing the complexity of the living environment by providing greater living space, additional cage mates, toys and access to running wheels. For decades it has been known that housing animals in EE for prolonged periods of time induces marked brain plasticity including changes in the thickness of cortical layers, increased complexity of dendrites, greater neuro- and gliogenesis, and changes in myelination (Henderson, 1970; Rosenzweig and Bennett, 1972; Rosenzweig, Bennett et al., 1972; Sirevaag and Greenough, 1985; Turner and Greenough, 1985; Nilsson, Perfilieva et al., 1999; van Praag, Kempermann et al., 2000). Further, EE can improve spatial learning and memory performance (Falkenberg, Mohammed et al., 1992; Leggio, Mandolesi et al., 2005). EE has been used extensively in the context of recovery from TBI where it can serve as an animal model for sustained cognitive and physical rehabilitation. Indeed, EE after TBI can attenuate the neuropathological and neuroinflammatory consequences of TBI as well as reducing functional deficits (Passineau, Green et al., 2001; Matter, Folweiler et al., 2011; Johnson, Traver et al., 2013). Further, EE can also alter sleep parameters and in particular has modulatory effects on REM sleep (Tagney, 1973; Gutwein and Fishbein, 1980; Kiyono, Seo et al., 1981). For instance, housing mice for 30 days in EE significantly increased the total time in REM sleep, number of REM sleep bouts and the percent of total sleep time spent in REM but without altering total sleep time (Gutwein and Fishbein, 1980). Although some studies have reported alterations in other sleep parameters, EE effects on REM sleep are the most consistently reported.

An important distinction has to be drawn between EE effects on spontaneous recovery and environmental plasticity (Giza and Prins, 2006). Although EE promotes functional recovery in animals injured during development, it fails to induce the developmental plasticity that can occur in the intact brain (Giza and Prins, 2006). For example, rats that underwent a lateral fluid percussion injury at 21 days of age did not exhibit EE-induced increase in cortical thickness, elaborated dendritic morphology or improvements in spatial learning and memory performance compared to sham-injured animals (Ip, Giza et al., 2002; Giza, Griesbach et al., 2005). This is also consistent with the observation that prenatal immune activation blocks EE-induced alterations in exploratory behavior and microglial physiology (Buschert, Sakalem et al., 2016). (Buschert, Sakalem et al., 2016). Here, prolonged housing in EE increased the amount of REM sleep in sham-injured animals but failed to modulate REM sleep in mice that were injured during development. The specific mechanisms of either EE-induced alterations in REM sleep (in Sham-injured mice) or the constraint on EE-induced REM changes (in injured mice) remain unspecified, although our IHC findings may

suggest a role for MCH neurons which are known to be critical modulators of REM sleep (e.g.,(Verret, Goutagny et al., 2003)). Although EE effects on REM sleep have been described previously, little mechanistic work has been conducted to dissect the neurological substrates. What we do know is that EE modulation of sleep physiology is only part of a large suite of changes that include changes in neuronal morphology, functional activation, behavior and cognition (van Praag, Kempermann et al., 2000).

In addition to measuring baseline sleep physiology (for both slow-wave and REM parameters) we also assessed the homeostatic sleep response to a brief period of sleep deprivation and did not detect any differential effects of injury or housing condition. Moreover, orexin immunoreactive cell numbers did not change following TBI. However, the number of pro-MCH immunoreactive cells in the lateral hypothalamus increased with environmental enrichment but only in mice that had not experienced traumatic brain injuries. This closely mirrors the sleep physiology as orexin inhibits REM sleep and MCH cell number was increased in the same group that increased REM sleep following enrichment. While we did not see a TBI-induced loss of hypothalamic cell populations as has been previously reported in some clinical studies and animal models of TBI (Baumann, Bassetti et al., 2008; Baumann, Bassetti et al., 2009; Willie, Lim et al., 2012), these observations were reported with more severe injuries and in adult animals and the model used here does not typically result in frank neuronal death. That said, the source of greater numbers of pro-MCH immunoreactive cells remains unspecified. MCH mRNA is detectable in the lateral hypothalamus by embryonic day 16, however, maturation of the system continues into adulthood with changes in neuronal morphology, axon density, and gene expression (Bittencourt, Presse et al., 1992; Presse, Hervieu et al., 1992; Steininger, Kilduff et al., 2004). Moreover, the period around weaning has been cited as a key time for maturation of the MCH system perhaps in the service of preparing the developing animal for the altered feeding patterns associated with solid food (Steininger, Kilduff et al., 2004). In any case, it seems possible that a traumatic brain injury, during this period of rapid neurodevelopment, could have persistent effects on MCH maturation and plasticity. To the best of our knowledge no group has examined the effects of environmental enrichment on MCH populations but an increase in MCH activity is consistent with the general pattern of increased REM sleep among animals housed in enriched environments. Future studies will investigate both the differential effects of enrichment on orexin- and MCH-positive neurons and the absence of plasticity in mice that had been previously injured. Importantly, further studies should characterize not only changes in neuron number, but activity (i.e., electrophysiological or calcium dynamic measurements) in response to injury and enrichment during development.

Although there were no overall differences in wakefulness across the treatment groups, sham-injured mice housed in EE exhibited greater locomotor activity both prior to and after sleep deprivation. Thus, one possibility is that TBI interfered with full participation in the EE condition and thus was unable to modulate sleep parameters. Although this remains a possibility we have shown previously that one-week of EE after a similar injury paradigm reduces axonal degeneration and normalizes gene expression of the plasticity and sleep-promoting molecule BDNF, indicating that this paradigm has significant modulatory effects in developmentally brain injured mice (Weil, Karelina et al., 2016). This does not rule out

the possibility that the increases in REM sleep are secondary to greater voluntary exercise in the SHAM-injured mice, however, voluntary exercise does not increase REM sleep (Lancel, Droste et al., 2003) and sleep parameters were recorded two weeks after the end of EE making it unlikely that there were direct effects of exercise on sleep. Unfortunately, the design of this study precluded directly measuring whether injury altered running behavior as the animals were housed with multiple individuals with access to the same running wheel. Thus, it remains unknown whether injured animals may have run less than sham-injured mice, which could help explain the lack of enrichment effects in injured mice. Future studies will investigate TBI-induced sleep adjustments during EE to determine whether an even larger response is evident when enrichment is ongoing. As this was an exploratory study, we did not conduct specific REM sleep deprivation experiments, which may further shed light on the plasticity of REM circuitry in injured animals and those exposed to environmental enrichment.

Although “standard” housing conditions are typically used as the comparison for enriched conditions it is not immediately obvious which condition best recapitulates free-living situations. This question is further complicated by the genetic lineage of laboratory rodents which have spent hundreds of generations living in captivity and being subject to genetic selection and founder effects (Sale, Berardi et al., 2014). Thus to a very real extent there is no “natural” environment for laboratory rodents. That aside, both standard and enriched laboratory conditions are likely impoverished compared to what a hypothetical free-living animal would face. Further, semi-natural outdoor enclosures produce greater cortical thickening than does laboratory-based EE (Rosenzweig, Bennett et al., 1972). Even so, none of these experimental procedures fully recapitulate the other challenges faced by free-living rodents including predation and the dangers of living near humans. In any case, the sleep response demonstrated in the mice housed in EE may better reflect the clinical picture. It therefore remains possible that rather than a constraint on plasticity in the sleep system EE may have revealed persistent deficits in REM sleep that are present in mice housed in standard laboratory conditions.

The injury paradigm that we have elected to use here is intended to recapitulate the effects of mild, but repeated closed-head injuries. This injury paradigm produces bilateral axonal degeneration and gliotic responses but does not induce detectable levels of frank neuronal death (Weil, Karelina et al., 2016; Karelina, Gaier et al., 2017; Karelina, Gaier et al., 2017). Silver-stained axons were detected at tissue collection in adult mice, indicating that degenerating axons remained chronically after injury. However, there was no effect of enrichment on axonal degeneration at this late time point. We previously showed that one-week of environmental enrichment, immediately after injury, could attenuate the axonal degeneration associated with this injury but it remains unclear whether any effect of enrichment on axon degeneration had resolved following prolonged recovery. In any case, this mild pathophysiological response fits with a comparatively small effect on sleep physiology that is only apparent in mice housed in enriched environments.

There are several important limitations that must be considered here. First, it remains possible that the implantation of the sleep recording device and required screws into the skull may have differentially produced inflammatory responses in mice with a history of

brain injury. Second, the study was not designed to determine whether these sleep changes that we observed in adulthood were also present earlier in life and thus future studies will need to investigate temporal changes in sleep physiology and changes following environmental enrichment.

In conclusion, the sleep phenotype we report here (among mice housed in standard conditions) is very mild and may reflect either the resolution of transient sleep dysfunction or an injury insufficient to produce permanent dysfunction in the sleep system. However, housing in EE revealed a key difference in REM sleep physiology such that enrichment significantly increased the amount of time spent in REM sleep only in sham-injured mice. Whether this reflects a constraint on plasticity caused by TBI or reveals the full sleep phenotype that was masked by housing in relatively impoverished environments remains unspecified. What is clear is that having a TBI, even a relatively mild one during juvenile development, persistently alters sleep responses into adulthood and indicates the critical importance of this problem in clinical populations where the consequences of sleep dysfunction can be severe.

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Abbreviations:

EE	environmental enrichment
EEG	electroencephalogram
EMG	electromyogram
mTBI	mild traumatic brain injury
NREM	non-rapid eye movement
PB	phosphate buffer
PBS	phosphate buffered saline
PFA	paraformaldehyde
REM	rapid eye movement
SD	sleep deprivation
SE	sham-injured mice housed in enriched environment
SS	sham-injured mice in standard housing
TBI	traumatic brain injury

TE	mTBI mice housed in enriched environment
TS	mTBI mice in standard housing

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Figure 1. Timeline of experimental procedures.

At postnatal day 21 all animals underwent either repeated closed head traumatic brain injuries or the sham procedure. Mild closed head injuries were performed and then repeated every other day for a total of three injuries. Immediately following injury animals were weaned to either standard laboratory conditions or into environmental enrichment for eight weeks. After the housing period, all animals were returned to standard conditions and the transmitter implantation surgery was performed. Following a two-week recovery period sleep physiology was recorded for 48 hours followed by a six-hour sleep deprivation and subsequent recovery.

Figure 2. Repeated mild TBI damages axons but does not alter orexin/hypocretin cell numbers in the lateral hypothalamus.

A-C) Representative silver stained axons in the corpus callosum. There was similar degeneration in mice housed in standard and enriched environments. Scale bar = 40 microns. D) Box plot of qualitative scoring of axon degeneration. An asterisk (*) indicates significantly greater axon degeneration in mTBI relative to Sham mice ($p < 0.05$). E-H) Representative images of orexin/hypocretin in the lateral hypothalamus. Scale bar=200 microns. I) Quantification of orexin/hypocretin cell immunoreactivity. J-M) Representative images of pro-melanin concentrating hormone staining in the lateral hypothalamus. Scale bar = 100 microns. Data are presented as means ($\pm$ SEM). An asterisk (*) indicates significant difference between indicated groups ($p < 0.05$). N=8–9/group.

Figure 3. Enrichment increases home cage activity in sham-injured but not TBI mice. Home cage activity during the sleep assessment revealed greater locomotor activity in the enriched mice, an effect that was mediated by the greater activity in the sham-injured animals. Data are presented as means ($\pm$ SEM). SS=sham/standard, TS=mTBI/standard, SE=sham/enriched, TE=TBI/enriched; SD = sleep deprivation. Zeitgeber time 0 indicates timing of lights on. N=7–9/group.

Figure 4. Enrichment enhances REM sleep in the sham-injured but not TBI mice.

A) Representative EEG and EMG traces depicting wake, non-REM and REM sleep. B) The percentage of time spent in REM sleep across the baseline sessions, sleep deprivation and recovery periods. C) Percentage of time spent in REM sleep averaged across baseline sessions for the day and night periods. D) Fourier transformation of EEG power spectrum during REM sleep. E) Number of bouts of REM sleep across the day. F) Percentage of time spent in Non-REM sleep across the baseline sessions, sleep deprivation and recovery periods. G) Percentage of time spent in non-REM sleep averaged across baseline sessions for the day and night periods. * Statistically different from standard-housed groups, # different from sham-standard. Data are presented as means ($\pm$ SEM). SS=sham/standard, TS=mTBI/standard, SE=sham/enriched, TE=TBI/enriched; SD = sleep deprivation. Zeitgeber time 0 indicates timing of lights on. N=7–9/group.