



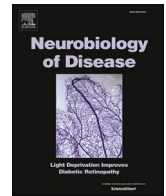
REM sleep suppression after penetrating traumatic brain injury is associated with alterations in the supramammillary–hippocampal circuit

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REM sleep suppression after penetrating traumatic brain injury is associated with alterations in the supramammillary–hippocampal circuit[☆]

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ABSTRACT

Sleep–wake disturbances are among the most prevalent and persistent consequences of traumatic brain injury (TBI), yet the circuit mechanisms underlying post-traumatic alterations in rapid eye movement sleep (REM) and associated cortical oscillations remain incompletely understood. Previous work has shown that REM-associated theta oscillations are determined by a Supramammillary (SuM)–hippocampal vesicular glutamate transporter 2 (Vglut2) circuit. First, we examined whether severe focal penetrating TBI (pTBI) suppresses REM and REM-associated theta power. Secondly, we examined whether these changes are associated with alterations in hypothalamic neuropeptide systems and SuM–hippocampal glutamatergic signaling. Adult male Sprague–Dawley rats (pTBI, $n = 7$; Sham, $n = 6$) were exposed to pTBI or sham procedures. After sham/TBI surgeries, EEG/EMG data was continuously sampled for 14 days by using telemetric implants, enabling analyses of sleep–wake states and cortical oscillations. At 14 days post injury, animals were sacrificed and brain tissue was sampled for immunohistochemical and in situ hybridization analyses. We analyzed orexin A (OXA) and melanin-concentrating hormone (MCH) in the lateral hypothalamus (LHA), Vglut2 in the SuM and hippocampus (CA2 and dentate gyrus), and nitric oxide synthase 1 (NOS1) in the SuM. pTBI resulted in reduced wakefulness during the light phase, increased non-REM sleep (NREM), and reduced REM. Power spectral density analyses of EEG data showed reduced theta (4–8 Hz) power during wake, REM, and NREM. Also, alpha (8–12 Hz) and sigma (12–16 Hz) reduced, in contrast to delta (1–4 Hz) power which increased across all vigilance states. Histological analyses showed reduced OXA and MCH in the LHA in pTBI animals. Similarly, Vglut2 transcript levels were reduced in the lateral SuM, and Vglut2 immunoreactivity was decreased in hippocampal CA2 and dentate gyrus. NOS1 expression in the SuM was not altered. Correlation analyses showed an association between the Vglut2 reduction in the SuM–hippocampal circuit and REM–theta oscillations. For the first time, we show that severe focal pTBI produces a sustained suppression of REM and REM associated theta oscillations, associated with decreased Vglut2 within the SuM–hippocampal circuit. These findings provide a framework linking SuM–hippocampal circuit dysfunction to post-traumatic REM sleep disturbances. Further research is needed to study potential causal effects of TBI on suppression of the SuM–hippocampal circuit and effects on hippocampal dependent cognitive functions.

1. Introduction

The incidence of traumatic brain injury (TBI) is increasing, constituting a substantial global burden (*Lancet Neurol.*, 2019). Common causes include traffic accidents, falls, sports-related concussions, and

combat-related injuries among military personnel. Morbidity following TBI is highly variable and injury dependent. However, sleep disturbances are common across injury types and severities, with up to 70% of patients reporting sleep-related problems regardless of injury type and severity (Grima et al., 2017; Orff et al., 2009). Given the critical role of

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sleep in maintaining both mental and physical health, understanding the pathophysiological mechanisms underlying sleep disturbances following TBI is an important focus in TBI research.

Sleep is divided into non-rapid eye movement sleep (NREM) and rapid eye movement sleep (REM). NREM is dominated by slow brain activity, indicated by the presence of delta waves (1–4 Hz) (Lee and Dan, 2012), and plays a critical role in physical restoration and cerebral recovery. In contrast, REM is dominated by a higher-frequency pattern theta waves (4–8 Hz) and plays a central role in emotional processing, memory formation and memory consolidation (Peever and Fuller, 2017). REM disturbances and associated cognitive deficits, such as anxiety, depression, and impaired memory (Grima et al., 2016; Blennow et al., 2016), are frequently reported following TBI. Disturbed REM sleep is also common in several neurodegenerative diseases, including Parkinson's disease, Lewy body dementia, and Alzheimer's disease (Elliott et al., 2024), all of which have been associated with neurotrauma.

Neural circuits regulating sleep, particularly REM sleep, are highly conserved across species (Peever and Fuller, 2017). The hypothalamus is a key region in this regulation. Wake-promoting neurons containing orexin A (OXA) (Dong et al., 2021) and REM-promoting neurons containing melanin-concentrating hormone (MCH) (Tortorello et al., 2015) are located in the lateral hypothalamus (LHA). Both neuronal populations are affected by TBI (Baumann et al., 2005; Baumann et al., 2009; Kousi et al., 2021; Valko et al., 2015).

Cortical oscillations have also been implicated in sleep disturbances observed after TBI (Pevzner et al., 2016). These oscillations arise from large populations of neurons that fire synchronously in repetitive patterns and are phylogenetically conserved. They are linked to specific anatomic regions and sleep–wake states (Buzsáki and Draguhn, 2004). For instance, theta oscillations reflect hippocampal activity during REM sleep (Buzsáki, 2002).

The supramammillary nucleus (SuM), located in the caudal hypothalamus, plays a central role in regulating theta oscillations (Renouard et al., 2015). It is divided into the medial SuM (SuMM) and the lateral SuM (SuML). SuML neurons expressing vesicular glutamate transporter 2 (Vglut2) project via the medial septum to CA2 and the dentate gyrus (DG) of the hippocampus. Vglut2 is essential for glutamate release from presynaptic terminals, where it is localized. This SuM–Hippocampal circuit contributes to REM-associated cortical theta oscillations (Renouard et al., 2015). Inhibition of SuM neurons co-expressing Vglut2 and nitric oxide synthase 1 (NOS1) reduces REM-associated theta power and wakefulness (Pedersen et al., 2017).

Several experimental TBI studies have reported alterations in sleep architecture, including changes in cortical oscillations and peptide levels in the LHA (Mountney et al., 2021; Thomasy et al., 2017; Skopin et al., 2015; Thomasy and Opp, 2019; Green et al., 2024; Korthas et al., 2022). However, the impact of TBI on the SuM–hippocampal circuit in relation to REM sleep and theta oscillations has not yet been studied.

Rodent TBI models provide valuable opportunities to investigate clinically relevant mechanisms of sleep regulation, as many sleep parameters are conserved across species. This study examined how a severe focal TBI model affects REM sleep and a putative neuroanatomical circuit involved in REM-theta oscillations. We hypothesized that pTBI would affect sleep microstructure more prominently than sleep macrostructure, and that these alterations would be associated with dysregulation of hypothalamic and SuM–hippocampal circuits involved in sleep-state regulation and REM-theta oscillations.

2. Methods

2.1. Animals and experimental design

A total of 13 male Sprague–Dawley rats (Janvier Labs, France, sham

$n = 6$, pTBI $n = 7$), weighing approximately 350 g at the start of the experiment, were included in this study.

To minimize animal use, this study was limited to male rats while still allowing the primary hypothesis to be tested without introducing additional biological variables, including sex and the estrous cycle, which would have required substantially larger group sizes.

Rats were single-housed in enriched cages under controlled environmental conditions (12:12 h light–dark cycle, lights on at 06:00; ambient temperature, 22 ± 0.5 °C; relative humidity 40–50%) with ad libitum access to food and water. After a one-week acclimatization period, animals were randomly assigned to TBI or sham procedures.

Following TBI or sham exposure, telemetric EEG and EMG recordings began on the first night (active phase) post-injury and continued uninterrupted for 14 nights and 13 days (inactive phase) (Fig. 1A). The experimental endpoint was set at two weeks post-injury to encompass the acute and subacute phases following TBI.

The primary outcomes were REM sleep duration and REM-associated theta power across the 14-day post-injury recording period. Secondary outcomes included wake and NREM sleep duration, spectral power across additional frequency bands, neuroinflammatory marker expression, lateral hypothalamic OXA and MCH levels, and Vglut2/NOS1-related measures in the hippocampus and SuM.

2.2. Surgical preparations

Each rat was anesthetized by intraperitoneal injection of 2.4 mL/kg of an anesthetic mixture consisting of fentanyl (5 mg/mL; 1 mL), midazolam (5 mg/mL; 1 mL), and ceptor (1 mg/mL; 0.5 mL). Following induction of anesthesia, animals were randomly assigned to either a pTBI or sham procedure. The fur over the surgical site was shaved, and ophthalmic ointment was applied to prevent corneal drying. The surgical area was then cleansed and disinfected by scrubs with sterile saline and chlorhexidine before surgery. During surgery, animals were placed in a stereotaxic frame on a temperature-controlled heating pad.

2.3. Experimental traumatic brain injury

The penetrating TBI model (pTBI) used in this study simulates a severe focal injury that is induced using a 2 mm wide, spherical-shaped rod penetrating the brain tissue of the left hemisphere. The impact occurs through a 2.8 mm wide craniotomy, positioned at 2.5 mm posterior and 2.5 mm lateral to bregma, at an impact speed of 90 m/s (Fig. 1B–C). Sham procedures were identical to pTBI procedures except that no rod penetration was performed. This is a well-characterized severe TBI model that produces a distinct focal injury and known behavioral sequelae. The injury causes sustained astrogliosis, apoptosis, and necrosis leading to cavity formation over weeks to months. Functional assessments have also been described, showing reference memory deficits, and altered sensorimotor function and anxiety levels (Plantman et al., 2012; Davidsson and Risling, 2016). Representative hematoxylin–eosin-stained sections from the injury level in sham and pTBI animals at 14 days post-injury is shown in Fig. 1G–I.

2.4. Implantation of telemetric EEG/EMG devices

Immediately after pTBI or sham procedures, the telemetric EEG/EMG device was implanted. A midline dorsal incision (4–6 cm) was made, and a subcutaneous pocket was created to position the transmitter with leads directed cranially.

Two EMG leads were inserted and secured into the trapezius musculature. To implant the EEG electrodes, two burr holes were drilled while keeping the dura intact. Stainless-steel screws (0–80 × 1/16; Bilaney, Düsseldorf, Germany) were inserted into the burr holes and the

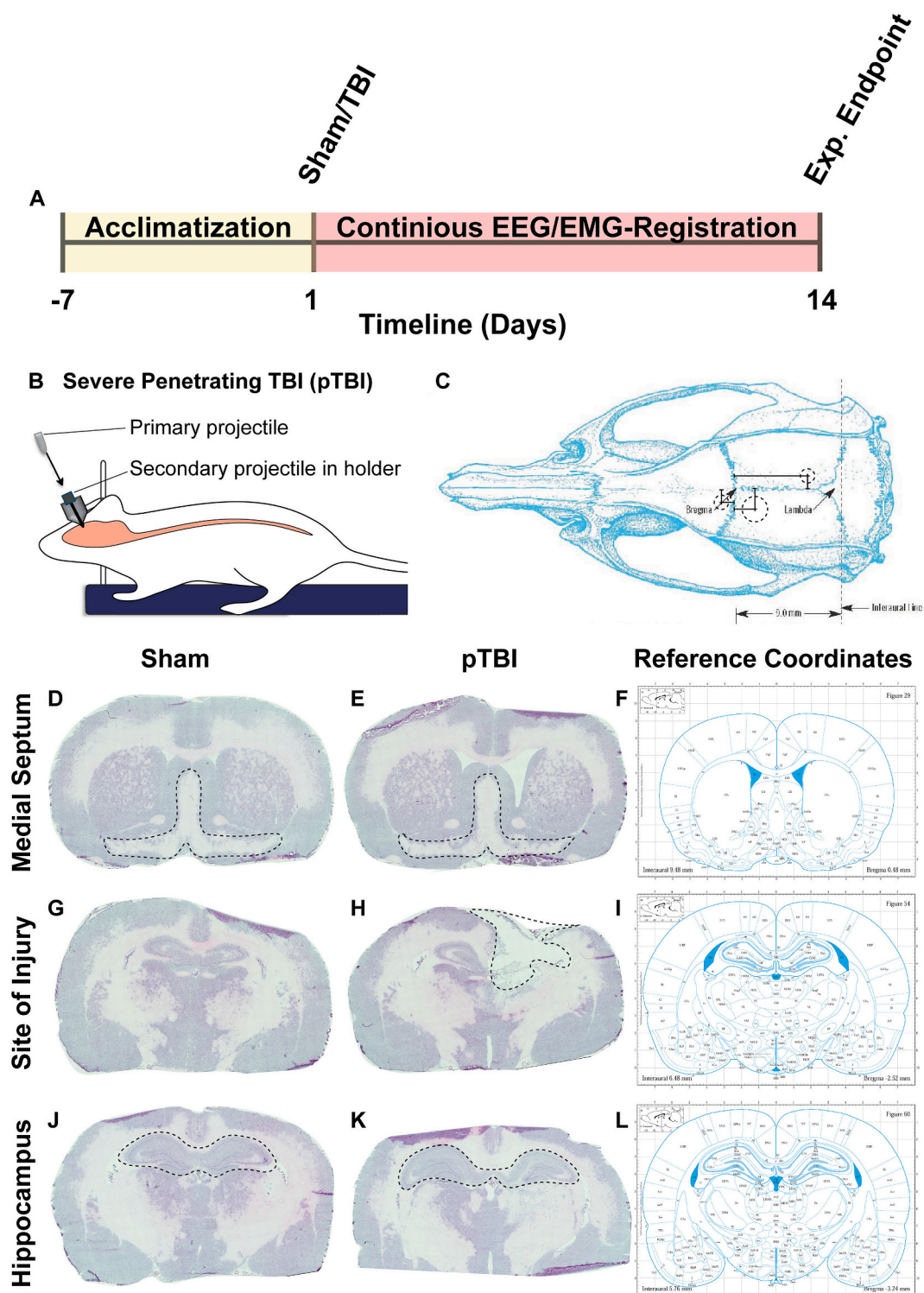


Fig. 1. Experimental design and histological verification of injury location in the penetrating TBI model.

(A) Timeline of the experimental protocol. (B) Schematic illustration of the penetrating traumatic brain injury model. (C) Dorsal skull view illustrating the location of the craniotomy and EEG electrode placement relative to bregma and lambda. (D–E) Coronal sections at the medial septum. Dashed outlines indicate the medial septum in Sham (D) and pTBI (E). (G–H) Representative coronal sections from the experimental endpoint (14 days post-injury) at the cortical injury level in Sham (G) and pTBI (H) animals. Dashed outlines indicate the lesion area. (J–K) Coronal sections at the dorsal hippocampus level in Sham (J) and pTBI (K) animals (caudal relative to D–F); dashed outlines indicate the analyzed hippocampal level. Reference coordinates for each level are shown (F), (I) and (L). All reference plates are adapted from Paxinos and Watson (2007).

EEG leads were wrapped around the screws. One electrode was positioned 2 mm anterior to bregma and the other 7 mm posterior to bregma. The electrodes were placed on opposite sides, 1.5 mm lateral to the sagittal suture (Fig. 1C). Once in place, the screws and leads were stabilized with insulating dental cement.

Following surgery, animals were returned to home cages which were placed on wireless receiver plates. Postsurgical buprenorphine was administered subcutaneously twice daily for three days post-surgery.

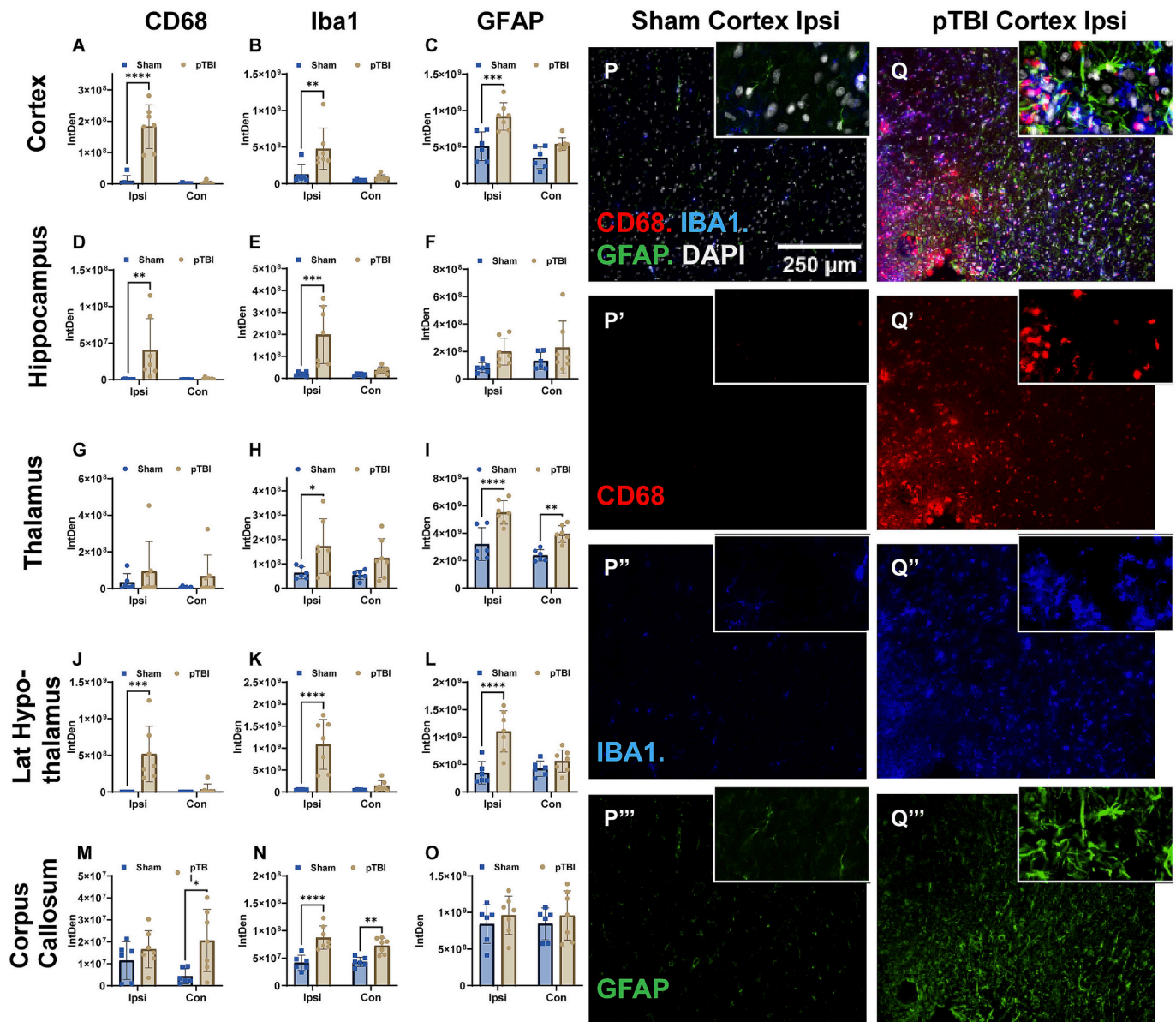
2.5. Tissue collection

Following anesthesia, brain samples were collected and snap-frozen on dry ice and stored at -70°C until sectioning. All animals from the sleep recording cohort contributed to tissue used in immunohistochemical (IHC) and fluorescence in situ hybridization (ISH) analyses. The brains were sectioned into serial coronal sections at $14\ \mu\text{m}$, using a Cryo-Star HM 560 M microtome (MICROM International GmbH, Heidelberg, Germany). Sections were cut at the levels of LHA (bregma -2.3

to $-3.8\ \text{mm}$), the dorsal hippocampus (bregma -3.0 to $-4.0\ \text{mm}$) and SuM (bregma -4.3 to $-5.3\ \text{mm}$) (Paxinos and Watson, 2007).

2.6. Immunohistochemistry

Slides were air-dried for 1 h at room temperature, fixed in 4% formaldehyde for 20 min, and rinsed three times in PBS ($3 \times 10\ \text{min}$). Sections were then blocked for 1 h in primary antibody buffer (0.01 M PBS, 0.1% NaN₃, 0.3% Triton X-100, 5% BSA) and incubated with primary antibodies for 24 h at 4°C . The following primary antibodies and dilutions were used: Vglut2 (Merck, AB2251-I; 1:500), OXA (Abcam, AB6214; 1:20,000), MCH (Phoenix Pharmaceuticals, ABH-070-47; 1:20,000), glial fibrillary acidic protein (GFAP; Fisher Scientific, AB13-0300; 1:1000), Iba1 (Thermo Fisher/Life Technologies, PA5-27436; 1:1000), CD68 (Serotec, MCA341R; 1:1000), synaptophysin (SynPhyS; Sigma, S5768; 1:1000), and neuronal nuclei marker (NeuN; Sigma, MAB377; 1:4000). After PBS washes ($3 \times 10\ \text{min}$), slides were incubated for 1 h at room temperature with secondary antibodies



(caption on next page)

Fig. 2. Severe focal pTBI induces region-specific neuroinflammatory responses in cortical and subcortical regions. Quantification of CD68 (A, D, G, J, M), Iba1 (B, E, H, K, N), and GFAP (C, F, I, L, O) integrated density in the cortex, hippocampus, thalamus, lateral hypothalamus, and corpus callosum following sham or pTBI. Data are presented as mean $\pm$ SD with individual animal values shown; sham $n = 6$, pTBI $n = 7$.

(A) Cortical CD68 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 32.79$, $p = 0.0001$; Hemisphere, $F(1, 11) = 44.31$, $p < 0.0001$; Hemisphere $\times$ Group, $F(1, 11) = 37.34$, $p < 0.0001$).
 (B) Cortical Iba1 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 7.837$, $p = 0.0173$; Hemisphere, $F(1, 11) = 17.09$, $p = 0.0017$; Hemisphere $\times$ Group, $F(1, 11) = 7.335$, $p = 0.0204$).
 (C) Cortical GFAP immunoreactivity was increased after pTBI (Group, $F(1, 11) = 19.72$, $p = 0.0010$; Hemisphere, $F(1, 11) = 21.50$, $p = 0.0007$; Hemisphere $\times$ Group, $F(1, 11) = 3.571$, $p = 0.0854$).
 (D) Hippocampal CD68 immunoreactivity was not significantly altered (Group, $F(1, 11) = 1.070$, $p = 0.3231$; Hemisphere, $F(1, 11) = 4.145$, $p = 0.0666$; Hemisphere $\times$ Group, $F(1, 11) = 0.0001210$, $p = 0.9914$).
 (E) Hippocampal Iba1 increased (Group $F(1, 11) = 4.958$, $p = 0.0478$; Hemisphere, $F(1, 11) = 14.64$, $p = 0.0028$; Hemisphere $\times$ Group, $F(1, 11) = 7.072$, $p = 0.0222$).
 (F) Hippocampal GFAP immunoreactivity was increased after pTBI, (Group, $F(1, 11) = 21.60$, $p = 0.0007$; Hemisphere, $F(1, 11) = 43.61$, $p < 0.0001$; Hemisphere $\times$ Group, $F(1, 11) = 4.300$, $p = 0.0624$).
 (G) Thalamic CD68 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 12.02$, $p = 0.0053$; Hemisphere, $F(1, 11) = 9.172$, $p = 0.0115$; Hemisphere $\times$ Group, $F(1, 11) = 9.173$, $p = 0.0115$).
 (H) Thalamic Iba1 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 25.90$, $p = 0.0003$; Hemisphere, $F(1, 11) = 13.91$, $p = 0.0033$; Hemisphere $\times$ Group, $F(1, 11) = 13.90$, $p = 0.0033$).
 (I) Thalamic GFAP immunoreactivity was increased after pTBI (Group $F(1, 11) = 22.27$, $p = 0.0006$; Hemisphere, $F(1, 11) = 4.985$, $p = 0.0473$; Hemisphere $\times$ Group, $F(1, 11) = 8.761$, $p = 0.0130$).
 (J) Lateral hypothalamic CD68 immunoreactivity showed a significant increase (Group, $F(1, 11) = 5.439$, $p = 0.0397$; Hemisphere, $F(1, 11) = 0.3133$, $p = 0.5869$; Hemisphere $\times$ Group, $F(1, 11) = 3.807$, $p = 0.0770$).
 (K) Lateral hypothalamic Iba1 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 23.64$, $p = 0.0005$; Hemisphere, $F(1, 11) = 3.424$, $p = 0.0913$; Hemisphere $\times$ Group, $F(1, 11) = 5.213$, $p = 0.0433$).
 (L) Lateral hypothalamic GFAP immunoreactivity was not significantly altered (Group, $F(1, 11) = 0.7992$, $p = 0.3905$; Hemisphere, $F(1, 11) = 0.0003026$, $p = 0.9864$; Hemisphere $\times$ Group, $F(1, 11) = 0.001760$, $p = 0.9673$).
 (M) Corpus callosum CD68 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 5.409$, $p = 0.0402$; Hemisphere, $F(1, 11) = 5.235$, $p = 0.0429$; Hemisphere $\times$ Group, $F(1, 11) = 5.111$, $p = 0.0450$).
 (N) Corpus callosum Iba1 immunoreactivity was increased after pTBI (Group, $F(1, 11) = 12.38$, $p = 0.0048$; effect of Hemisphere, $F(1, 11) = 10.28$, $p = 0.0084$; Hemisphere $\times$ Group, $F(1, 11) = 9.577$, $p = 0.0102$).
 (O) Corpus callosum GFAP immunoreactivity was not significantly altered (Group, $F(1, 11) = 4.140$, $p = 0.0667$; Hemisphere, $F(1, 11) = 1.017$, $p = 0.3348$; Hemisphere $\times$ Group, $F(1, 11) = 0.04899$, $p = 0.8289$).

Statistical significance for post-hoc comparisons is indicated in the graphs as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

(P–P') Representative immunofluorescence images from sham ipsilateral cortex, and (Q–Q') pTBI ipsilateral cortex. Insets show higher-magnification views highlighting cellular morphology and marker distribution. CD68 is shown in red, Iba1 in blue, GFAP in green, and DAPI in white. Scale bar: 250 μ m.

diluted in secondary buffer (0.01 M PBS, 0.1% NaNa, 0.3% Triton X-100): donkey anti-mouse Alexa Fluor 488 (Thermo Fisher, A10037), donkey anti-rabbit Alexa Fluor 555 (Life Technologies, A-31572), and donkey anti-mouse Alexa Fluor 647 (Life Technologies, A-31571). All secondary antibodies were diluted 1:500. Sections were counterstained with DAPI (5 min), and coverslips were mounted using ProLong Diamond Antifade Mountant (Life Technologies, P36961).

2.7. Fluorescence in situ hybridization

FISH was performed on sections adjacent to those used for IHC using the ViewRNA™ Multiplex Assay Kit (Thermo Fisher Scientific, QVT4800). Due to section availability, only three sections per animal were used for FISH. No animals were excluded due to section availability or tissue quality. Slides were transferred directly from -80°C storage into ice-cold 4% formaldehyde and fixed for 19 h at 4°C , then rinsed briefly in PBS and air-dried for 30 min at room temperature. Sections were incubated with protease (1:100 in pre-warmed PBS) for 10 min at 40°C , washed, post-fixed in 4% formaldehyde for 5 min, and subsequently hybridized with target-specific mRNA probes. The following probes and dilutions were used: Hcrt (VC6-3230116-VCP; 1:500), Pmch (VC1-3059433-VT; 1:500), Vglut2 (VC6-15120-VC; 1:500) and NOS1 (VC1-11565-VT; 1:500). Pro-melanin-concentrating hormone (PMCH) is the precursor transcript for MCH. Peptidylprolyl isomerase B (Ppib), also known as Cyclophilin B, is a housekeeping gene and was used as a positive technical control. Hybridization was performed for 2 h at 40°C , after which slides were incubated sequentially with Pre-Amplifier, Amplifier, and Label Probe mixes, each for 30 min at 40°C . The fluorescent label probes used were Label probe type 1 Alexa Fluor 647, type 4 Alexa Fluor 488 and type 6 Alexa Fluor 555, all diluted 1:25. Finally,

slides were counterstained with DAPI (1:100) and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific).

2.8. Sleep assessment

Sleep data were analyzed in NeuroScore (Data Sciences International, DSI) based on EEG, EMG, and activity signals from telemetry implants. The automated sleep scoring algorithm is well established and has also been used by other groups (Dautan et al., 2026). Each 10-s epoch of the whole experimental timeline was automatically scored into Wake, NREM, or REM. Sleep scoring was then visually verified, and when incorrect classifications were identified, the algorithm parameters for that specific subject were adjusted to ensure accurate scoring.

Three parameters were used for the automatic sleep scoring: delta power, theta power, and EMG amplitude. On average, a delta ratio of 0.5 corresponded to the highest probability of REM/Wake, whereas a ratio of 1.0 indicated NREM. A theta-to-delta ratio of 1.3 was associated with Wake/NREM, while 3.0 reflected the highest probability of REM. For EMG power, an average ratio of 0.7 indicated REM/NREM, and 1.8 corresponded to wake. To optimize accuracy of REM-scoring, EEG frequency bands were defined as delta (0.5–4 Hz) and theta (6–9 Hz). Thus, a narrower and slightly higher theta range was used for sleep scoring, whereas the standard theta frequency range (4–8 Hz) was used for the spectral power analyses.

Epochs in which both EEG and EMG signals exceeded 1 mV were classified as artifacts and excluded from further analysis. Data were segmented into 12-h intervals. These corresponded to the light phase, defined as the inactive period, and the dark phase, defined as the active period, beginning with the first dark phase after surgery. If sleep recording for an individual animal was interrupted because of

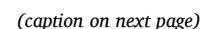


Fig. 3. Longitudinal changes in sleep–wake states and spectral power following pTBI. Time-course analysis of sleep–wake architecture in sham and pTBI animals during the day (inactive) and night (active) phases. (A–B) Total wake time. (C, D) Relative theta power during wake. (E, F) Total REM sleep. (G, H) Relative theta power during REM sleep. (I, J) Total NREM sleep. (K, L) Relative theta power during NREM sleep.

Data are presented as mean $\pm$ SD, with shaded areas indicating SD. Full F statistics, degrees of freedom, and *p*-values for all fixed effects and significant post hoc analyses are provided in Supplementary Figs. 1–3.

(M–N) Heatmap summaries of spectral power ratios (pTBI/sham) across frequency bands are shown for the light (M) and dark (N) phases during Wake, REM, and NREM states. Values >1 indicate higher power in pTBI relative to sham. Red boxes denote significant increases, green boxes significant decreases, and yellow boxes denote no significant change in the pTBI/Sham power ratio within each frequency band. Arrows indicate increase, decrease or stationary changes. Full statistical details for the spectral power ratio analyses are provided in Supplementary Figs. 4–10.

(O–P) Representative examples of EEG/EMG recordings, hypnograms, and power spectral density plots from sham and pTBI animals during the day and night. Color intensity represents spectral power across frequencies over time. Red arrows highlight activity within the theta frequency band.

unexpected technical issues, that animal was excluded from the corresponding 12-h period. These exclusions are summarized in Supplementary Table 1.

For each vigilance state, power spectral density was calculated from the EEG signal, sampled at 250 Hz, using a 10th-order Fast Fourier Transform (FFT), which provides a frequency resolution of 0.244 Hz. Relative band power was calculated as the ratio between power in the frequency band of interest and total signal power. Power bands were analyzed within the delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), sigma (12–16 Hz), beta (16–24 Hz), low gamma (24–49 Hz) and high gamma (51–80 Hz) ranges. Given that the 8–12 Hz range in rats has been described as overlapping with the upper portion of the theta band rather than representing a canonical human-like alpha rhythm, the term “alpha” in the present study should be interpreted as a nominal frequency-band label. Specifically, the 4–8 Hz band is referred to as theta, whereas the 8–12 Hz band should be considered an adjacent upper-theta/nominal alpha range within a broader rat theta-related spectrum. The resulting periodogram was exported to Excel, where mean power within each predefined frequency band was calculated for each vigilance state.

2.9. Microscopy and image analysis

Fluorescence imaging was performed on a Nikon Eclipse Ni-E microscope equipped with an Andor Zyla camera, using NIS Elements software. For each region of interest (ROI), 3–8 sections were randomly selected for imaging at 10 $\times$ or 20 $\times$ magnification using FITC, DAPI, and Texas Red filters, with identical imaging parameters applied across all experimental groups. The exact number of sections used for each staining and each ROI is presented in Supplementary Table 2. For confocal colocalization analysis, images were acquired using a Carl Zeiss LSM 880, Oberkochen, Germany, with a 60 $\times$ magnification. Z-stack imaging was performed, and stack projections were generated using FIJI.

ROIs were defined according to the anatomical borders of the LHA, SuMM, SuML, hippocampal cornu ammonis (CA) subregions, and dentate gyrus (DG). For each analyzed peptide or mRNA, the exposure time was set based on the intensity of the strongest positive signal and then kept constant across all slides. Blurry images, images with bubbles or images from an incorrect level of ROI were excluded. Quantification was performed in FIJI by calculating the integrated density (IntDen), defined as the product of the total positive area and the mean intensity of the positive signal. All regions were analyzed bilaterally. For SuM, SuMM and SuML were analyzed separately.

2.10. Statistical analysis

At the conclusion of the study, the final group sizes were pTBI, *n* = 7, and sham, *n* = 6, for a total of 13 animals. All animals contributed to sleep and histological analyses. Statistical analyses were conducted using GraphPad Prism version 9 (GraphPad Software, CA, USA), with α set to 0.05.

Longitudinal sleep data were analyzed using mixed-effects models with restricted maximum likelihood estimation (REML). The model included Group (pTBI vs. sham), Day (1–14 post-injury), and the Group $\times$ Day interaction as fixed effects, with Subject modeled as a random effect to account for within-subject correlations across repeated measurements. Results are reported in the figure legends as *F* (df numerator, df denominator) = *F* value, *p* value for each fixed effect. Separate analyses were conducted for the light phase (06:00–18:00) and dark phase (18:00–06:00), corresponding to 12-h bins across the 24-h cycle. Šidák-corrected post-hoc comparisons were performed to identify group differences within individual days whenever the REML analysis showed a significant effect of Group or Group $\times$ Day interaction. REML was chosen over repeated-measures ANOVA because it accommodates missing data and accounts for the repeated-measures structure of the longitudinal recordings.

For histological analyses of bilateral structures, data were analyzed using two-way repeated-measures ANOVA with Group as the between-subject factor and Hemisphere as the within-subject factor and animals matched across hemispheres. Significant effects were followed by Šidák-corrected post-hoc comparisons, comparing pTBI and sham animals within each hemisphere. For non-bilateral analyses, Welch's *t*-test was used to account for unequal variances, which could not be assumed.

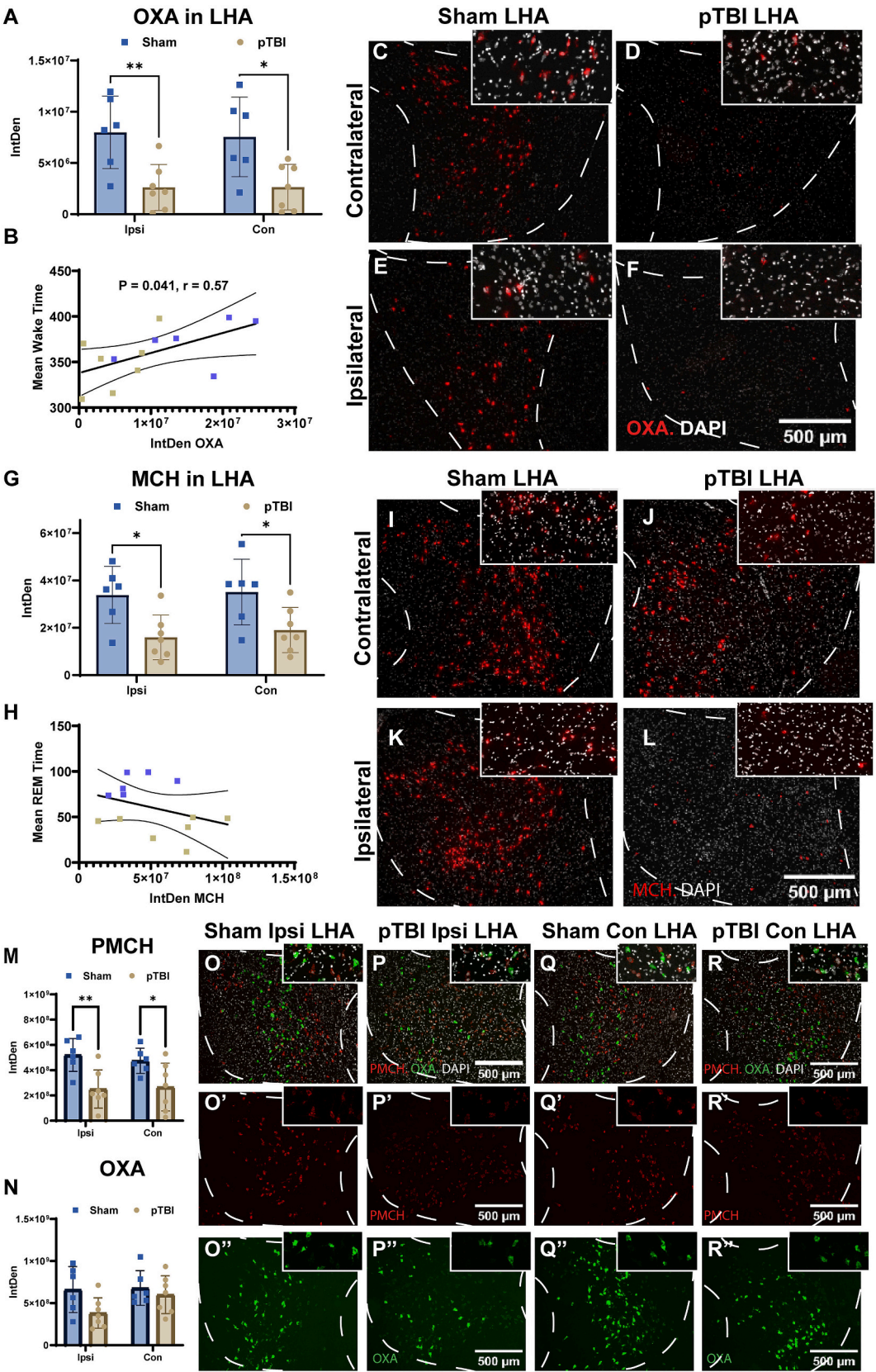
Pearson's correlations were performed to assess associations between histological measures and telemetric outcomes. No animals were excluded from any histological analyses due to tissue quality or section availability, and the correlation analyses were based on all 13 animals. However, individual images or time points were excluded when technical issues were present, such as poor image quality, bubbles or incorrect anatomical level.

Statistical significance for post-hoc comparisons is indicated in the graphs as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

3. Results

3.1. pTBI induces inflammation and gliosis in cortical and subcortical structures

IHC analyses demonstrated robust region- and hemisphere-specific glial responses following pTBI (Fig. 2). Overall, contralateral regions showed smaller or non-significant changes across most structures analyzed at the experimental endpoint. In the ipsilateral cortex adjacent to the penetration injury, spanning layers I–VI, pTBI animals showed significantly increased immunoreactivity of CD68 and Iba1 compared with sham controls (Fig. 2A–B). This was accompanied by elevated GFAP IntDen (Fig. 2C). In the hippocampus, CD68 and Iba1 immunoreactivity showed modest increases, but did not reach significance (Fig. 2D–E), whereas GFAP IntDen was significantly elevated (Fig. 2F). In the ipsilateral thalamus, CD68 and Iba1 levels were markedly increased (Fig. 2G–H), with a parallel increase in GFAP expression (Fig. 2I). The LHA showed increased CD68 and Iba1 immunoreactivity, most prominently for Iba1, whereas GFAP IntDen was not significantly increased (Fig. 2J–L). A similar pattern was observed in the corpus



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Fig. 4. Alterations in OXA and MCH signaling in the lateral hypothalamic area following severe focal pTBI. (A) IHC quantification of OXA in the LHA in sham and pTBI animals showed a significant reduction in OXA IntDen, $n = 6-7$ (Group, $F(1, 11) = 10.15$, $p = 0.0087$; Hemisphere, $F(1, 11) = 0.2253$, $p = 0.6443$; Hemisphere $\times$ Group, $F(1, 11) = 0.2904$, $p = 0.6007$). (B) Correlation between OXA IntDen and mean wake time is shown. Pearson correlation $p = 0.0403$, $r = 0.5739$, 95% confidence interval 0.03349 to 0.8546 and $n = 13$. (C, D) Representative images of OXA immunofluorescence in contralateral and (E, F) ipsilateral LHA from sham and pTBI animals are shown with higher-magnification insets. (G) Quantification of MCH IntDen in the LHA. Two-way ANOVA with Šídák-corrected post-hoc comparisons showed a significant reduction in MCH IntDen, $n = 6-7$ /group (Group, $F(1, 11) = 7.634$, $p = 0.0185$; Hemisphere, $F(1, 11) = 3.311$, $p = 0.0961$; Hemisphere $\times$ Group, $F(1, 11) = 0.6149$, $p = 0.4495$). (H) Correlation between MCH IntDen and mean REM time; Pearson correlation, $r = -0.3492$, $p = 0.2421$, 95% confidence interval -0.7550 to 0.2498 and $n = 13$. Representative images of MCH staining in contralateral (K, L) and ipsilateral LHA from sham and pTBI animals are shown with corresponding higher-magnification insets. (M-N) Quantification of PMCH and OXA mRNA levels in ipsilateral and contralateral LHA. Two-way ANOVA with Šídák-corrected post-hoc comparisons showed significant reduction in PMCH transcript levels, $n = 6-7$ /group, (Group, $F(1, 11) = 9.305$, $p = 0.0110$; Hemisphere, $F(1, 11) = 0.3617$, $p = 0.5597$; Hemisphere $\times$ Group, $F(1, 11) = 1.362$, $p = 0.2679$), whereas OXA transcript levels were not significantly altered, $n = 6-7$ /group, (Group, $F(1, 11) = 2.827$, $p = 0.1208$; Hemisphere, $F(1, 11) = 3.572$, $p = 0.0854$; Hemisphere $\times$ Group, $F(1, 11) = 2.523$, $p = 0.1405$). (O-R) Representative double-labeled FISH images of PMCH/OXA in ipsilateral and contralateral LHA from sham and pTBI animals are shown. Statistical significance for Šídák-corrected post-hoc comparisons is indicated as $*p < 0.05$ and $**p < 0.01$.

callosum, where pTBI selectively increased the IntDen of CD68 and Iba1, whereas GFAP IntDen changes were limited (Fig. 2M–O). Representative staining images are presented in Fig. 2, showing CD68-, Iba1-, and GFAP-positive cells in the ipsilateral cortex of sham animals (Fig. 2P) and pTBI animals (Fig. 2Q).

3.2. pTBI induces pronounced alterations in the macro- and microstructure of sleep–wake phases

pTBI altered sleep–wake architecture across the 14-day recording period (Fig. 3). During the day, corresponding to the inactive phase, total wake time was significantly reduced in pTBI animals compared with sham controls, with a significant main effect of Group, $F(1, 11) = 5.000$, $p = 0.0470$, while the effects of Time and Time $\times$ Group interaction were not significant (Fig. 3A). Wake duration during the night, corresponding to the active phase, did not differ between groups, $F(1, 11) = 0.2275$, $p = 0.6427$, although there was a significant effect of Time, $F(3.837, 41.91) = 4.264$, $p = 0.0061$ (Fig. 3B). Total REM sleep was markedly reduced in pTBI animals during both day and night, with significant main effects of Group during the day, $F(1, 11) = 25.53$, $p = 0.0004$, and night, $F(1, 11) = 38.95$, $p < 0.0001$ (Fig. 3E–F). During the initial days and nights, some pTBI animals exhibited 0 min of REM sleep, indicating a complete absence of REM during those 12-h periods (Supplementary Fig. 2A–B). In contrast, total NREM sleep was significantly increased only during the day, $F(1, 11) = 11.88$, $p = 0.0055$ (Fig. 3I), while no significant group difference was observed during the night, $F(1, 11) = 2.661$, $p = 0.1311$ (Fig. 3J).

During the initial post-surgical periods, we observed an increase in NREM in both pTBI, and sham animals accompanied by slight decreases in REM sleep and total wake time during both day and night.

Power spectral analyses revealed state- and phase-specific changes in oscillatory activity. During wake, relative theta power was significantly reduced in the light phase, $F(1, 11) = 6.056$, $p = 0.0316$ (Fig. 3C), with a non-significant trend during the dark phase, $F(1, 11) = 3.566$, $p = 0.0856$ (Fig. 3D). pTBI animals exhibited a significant reduction in theta power during REM sleep, with significant main effects of Group during the day, $F(1, 11) = 24.73$, $p = 0.000$ (Fig. 3G) and night $F(1, 11) = 27.32$, $p = 0.0003$ (Fig. 3H). During NREM sleep, theta power showed a modest but statistically significant reduction in both phases, with significant main effects of Group during the light phase, $F(1, 11) = 5.907$, $p = 0.0334$ (Fig. 3K), and dark phase, $F(1, 11) = 7.213$, $p = 0.0212$ (Fig. 3L). When total REM sleep was 0 min, REM-theta power could not be calculated and was treated as missing. The reductions in REM sleep and REM-theta power remained significant after excluding the early post-surgical analgesia window (Supplementary Fig. 2C–D/G–H). Full fixed-effect statistics for Time, Group, and the Time $\times$ Group interaction for all mixed-effects models are provided in Supplementary Figs. 1–3. Red arrows indicate theta power during REM in the power spectral

density plots in Fig. 3O–P.

In addition, we also observed alterations in other frequency bands across the study period (Supplementary Figs. 4–9). Summary of TBI/sham spectral power ratios over the whole recording time are presented in Fig. 3M–N. Delta power increased across all vigilance states during both day and night (Supplementary Fig. 4A–F). In contrast, and like theta, alpha power decreased across all vigilance states at both day and night (Supplementary Fig. 5A–F). Sigma power decreased across all vigilance states during the night, but only during Wake and NREM sleep during the day (Supplementary Fig. 6A–F). Beta power was reduced only during NREM sleep, across days and nights (Supplementary Fig. 7A–F). Low gamma power increased during REM sleep during both day and night (Supplementary Fig. 8A–F), whereas high gamma was not significantly altered (Supplementary Fig. 9A–F). EMG amplitude was not affected by pTBI (Supplementary Fig. 10A–F).

3.3. Sleep–wake disruption and concomitant reduction of OXA and MCH levels in the LHA

Quantification of OXA peptide levels in the LHA demonstrated significant reductions following pTBI (Fig. 4A). OXA levels were significantly decreased in both hemispheres of pTBI animals compared with sham controls. Correlation analysis revealed a positive association between OXA integrated density and mean wake time (Fig. 4B). Representative immunofluorescence images illustrate reduced OXA levels in the LHA of pTBI animals relative to sham controls in both hemispheres (Fig. 4C–F).

MCH peptide levels in the LHA were also significantly reduced following pTBI (Fig. 4G). Ipsilateral MCH integrated density was lower in pTBI animals compared with sham controls, whereas contralateral differences were less pronounced and did not reach statistical significance. MCH integrated density did not correlate significantly with mean REM sleep duration (Fig. 4H).

FISH analysis of PMCH demonstrated a significant reduction in the ipsilateral LHA of pTBI animals (Fig. 4M). OXA transcript quantification in the same double-labeled sections showed a similar trend but no significant group difference (Fig. 4N).

3.4. Decreased hippocampal Vglut2 levels correlate with REM-theta suppression

In the CA2, Vglut2 integrated density was significantly decreased in both hemispheres of pTBI animals compared with sham controls (Fig. 5A). The same effect was observed in the DG, where ipsilateral and contralateral Vglut2 expression was significantly lower in pTBI animals compared with sham controls (Fig. 5C).

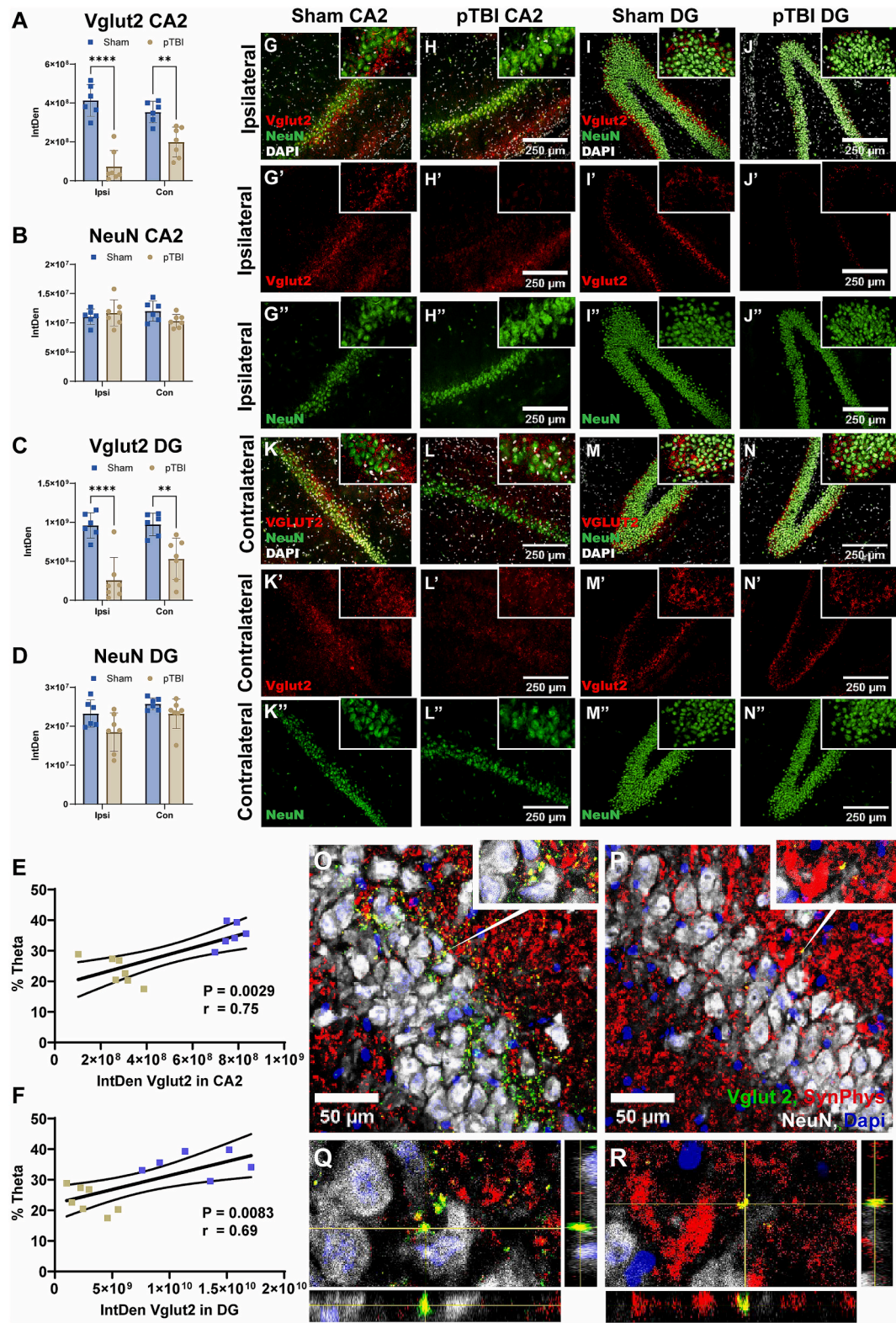
NeuN IntDen in CA2 did not differ between groups (Fig. 5B). In the DG however, NeuN IntDen showed a significant hemisphere effect and a

trend toward a group effect, but the main effect of Group x Hemisphere did not reach statistical significance (Fig. 5D).

Importantly, even when comparing the Vglut2 integrated density normalized to NeuN, the observed differences in both CA2 and DG remained significant (Supplementary Fig. 11A-B).

Correlation analyses demonstrated positive associations between Vglut2 integrated density and relative theta power. In CA2, Vglut2 levels correlated with relative theta power during REM sleep (Fig. 5E). A similar positive correlation was observed in the DG (Fig. 5F).

To further assess the spatial distribution of Vglut2 labeling,



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Fig. 5. Reduced Vglut2 levels in the hippocampal CA2 and the dentate gyrus following severe focal pTBI. Quantification of Vglut2 immunoreactivity expressed as integrated density in CA2 (A) and DG (C). NeuN immunoreactivity in CA2 (B) and DG (D), in ipsilateral and contralateral hemispheres of sham and pTBI animals. Data are presented as mean $\pm$ SD with individual data points shown ($n = 6$ sham ipsilateral, $n = 7$ pTBI ipsilateral, $n = 6$ sham contralateral, $n = 7$ pTBI contralateral).

(A) Vglut2 levels were reduced in CA2 (Group, $F(1, 11) = 151.4, p < 0.0001$; Hemisphere, $F(1, 11) = 0.8018, p = 0.3897$; Hemisphere $\times$ Group, $F(1, 11) = 6.324, p = 0.0287$).

(B) NeuN in CA2 did not differ between sham and pTBI (Group, $F(1, 11) = 0.6343, p = 0.4426$; Hemisphere, $F(1, 11) = 0.1205, p = 0.7351$; Hemisphere $\times$ Group, $F(1, 11) = 3.348, p = 0.0945$).

(C) Vglut2 levels in DG were also reduced, (Group, $F(1, 11) = 27.53, p = 0.0003$; effect of Hemisphere, $F(1, 11) = 4.573, p = 0.0557$; Hemisphere $\times$ Group, $F(1, 11) = 3.733, p = 0.0795$).

(D) NeuN in DG showed a modest effect of hemisphere (Group, $F(1, 11) = 4.161, p = 0.0661$; Hemisphere, $F(1, 11) = 11.63, p = 0.0058$; Hemisphere $\times$ Group, $F(1, 11) = 1.119, p = 0.3128$).

(E) Pearson correlation analysis showed positive associations between relative theta power and Vglut2 immunoreactivity in CA2 with $r = 0.6062, p = 0.0281$, 95% confidence interval 0.08297 to 0.8675, $n = 13$.

(F) Pearson correlation analysis also revealed a positive association between relative theta power and Vglut2 immunoreactivity in DG with $r = 0.6957, p = 0.0083$, 95% confidence interval 0.2347 to 0.9012 and $n = 13$.

Statistical significance for post-hoc comparisons is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

(G–N) Representative immunofluorescence images. (G, H) Ipsilateral hippocampal CA2 in sham and pTBI animals. (I, J) DG in sham and pTBI animals. Corresponding single-channel views are shown below each multichannel image. (K, L) Contralateral hippocampal CA2 in sham and pTBI animals. (M, N) DG in sham and pTBI animals. Insets show higher magnification views.

(O–R) Representative confocal images showing co-labeling of Vglut2 with the presynaptic marker synaptophysin in the ipsilateral CA2 of the hippocampus in sham (O) and pTBI (P). Higher-magnification orthogonal views from sham in (Q) and pTBI in (R). These images illustrate the punctate, synaptic distribution of Vglut2 signal in relation to NeuN-positive cells.

representative confocal images were added showing Vglut2 in relation to the presynaptic marker synaptophysin, NeuN-positive neurons, and DAPI (Fig. 5O–R). These images demonstrate a punctate Vglut2 signal co-localized with synaptophysin-positive structures, consistent with a presynaptic distribution of the Vglut2 in the hippocampus.

3.5. Decreased Vglut2 transcript levels in the SuM link REM-theta suppression to SuM-hippocampal alterations in glutamatergic signaling

Quantification of NOS1 and Vglut2 transcript levels in the SuM revealed subdivision-specific effects following pTBI (Fig. 6). NOS1 integrated density in the SuML and SuMM subdivisions did not differ between sham and pTBI animals (Fig. 6A–B). Correlation analysis showed no significant association between total NOS1 expression in the SuM and relative theta power during REM sleep (Fig. 6C).

Unlike NOS1, Vglut2 transcript levels showed a significant reduction in the ipsilateral SuML and a non-significant trend in the contralateral SuML of pTBI animals compared with sham controls (Fig. 6D). Vglut2 levels in the SuMM did not differ between groups (Fig. 6E). Total Vglut2 integrated density in the SuM showed a positive correlation with relative theta power during REM sleep (Fig. 6F).

4. Discussion

4.1. pTBI suppresses REM sleep and disrupts SuM-hippocampal theta signaling

In this study, we investigated REM sleep, general sleep architecture and the SuM-hippocampal circuit regulating REM-theta oscillations in the context of TBI. We used a severe focal penetrating TBI model that causes a well-characterized, anatomically distinct injury and known behavioral sequelae. We acquired continuous EEG/EMG recordings with per-vigilance-state spectral analysis across day and night, providing a comprehensive longitudinal dataset, and assessed within-animal histological correlations, rather than relying on cross-cohort inference. This approach, which is not commonly employed in experimental TBI-sleep research, adds depth to the histological data and correlation analyses, despite the moderate number of biological replicates.

We observed pronounced alterations in both sleep architecture and REM-associated theta oscillations. Injured animals exhibited reduced wakefulness during the day, corresponding to the inactive phase, increased NREM sleep, and marked suppression of REM sleep across the 14-day recording period post-injury. REM sleep suppression was

accompanied by a robust reduction in theta power and additional alterations across frequency bands and vigilance states.

At the circuit level, we observed significant reductions in OXA and MCH levels in the LHA. Beyond the LHA, Vglut2 levels were reduced in the hippocampal CA2, the dentate gyrus and in the SuML. Vglut2 levels in both the hippocampus and the SuM correlated with REM-theta power, whereas NOS1-expressing neurons in the SuM were preserved. Together, these findings suggest that pTBI is associated with altered glutamatergic signaling within the SuM-hippocampal network, providing a potential circuit-level correlate of REM-theta suppression. Given the established role of REM-theta oscillations in memory and emotional regulation, disruption of this pathway may contribute to the cognitive and affective sequelae observed after TBI. (See Fig. 7).

4.2. Concurrent OXA and MCH reductions suggest disrupted hypothalamic homeostasis after TBI

Alterations in the OXA and MCH systems have previously been described after TBI in humans (Baumann et al., 2005; Baumann et al., 2009; Kousi et al., 2021; Valko et al., 2015; Baumann et al., 2007; Castriotta and Murthy, 2011; Zhang et al., 2024) and rodents (Thomasy et al., 2017; Thomasy and Opp, 2019; Somach et al., 2023). However, the present study is, to our knowledge, the first to link post-traumatic REM-theta suppression to disrupted SuM-hippocampal glutamatergic signaling. The partial mismatch between OXA peptide- and transcript-level findings may reflect limited statistical power, temporal differences between transcriptional and peptide-level regulation, or post-transcriptional mechanisms. The concurrent decrease in OXA and MCH, representing opposing axes of wake-REM regulation, suggests a disturbance in the hypothalamic homeostasis rather than a unidirectional loss of arousal drive. This dual impairment may contribute to the mixed sleep phenotypes frequently seen in patients with TBI, where hypersomnia and insomnia coexist. Additionally, given the broader roles of OXA including motivation, appetite, homeostasis (Ganjavi and Shapiro, 2007) and MCH, including i.e., appetite, stress, memory, (Tortorolo et al., 2015; Adamantidis and de Lecea, 2009; Hervieu, 2003), these OXA and MCH changes may also be linked to known symptoms such as changes in appetite, weight, motivation, and memory following TBI.

4.3. Interpretation of the supramammillary-hippocampal Vglut2 in pTBI

The SuM regulates cortical theta oscillations indirectly (Vertes,

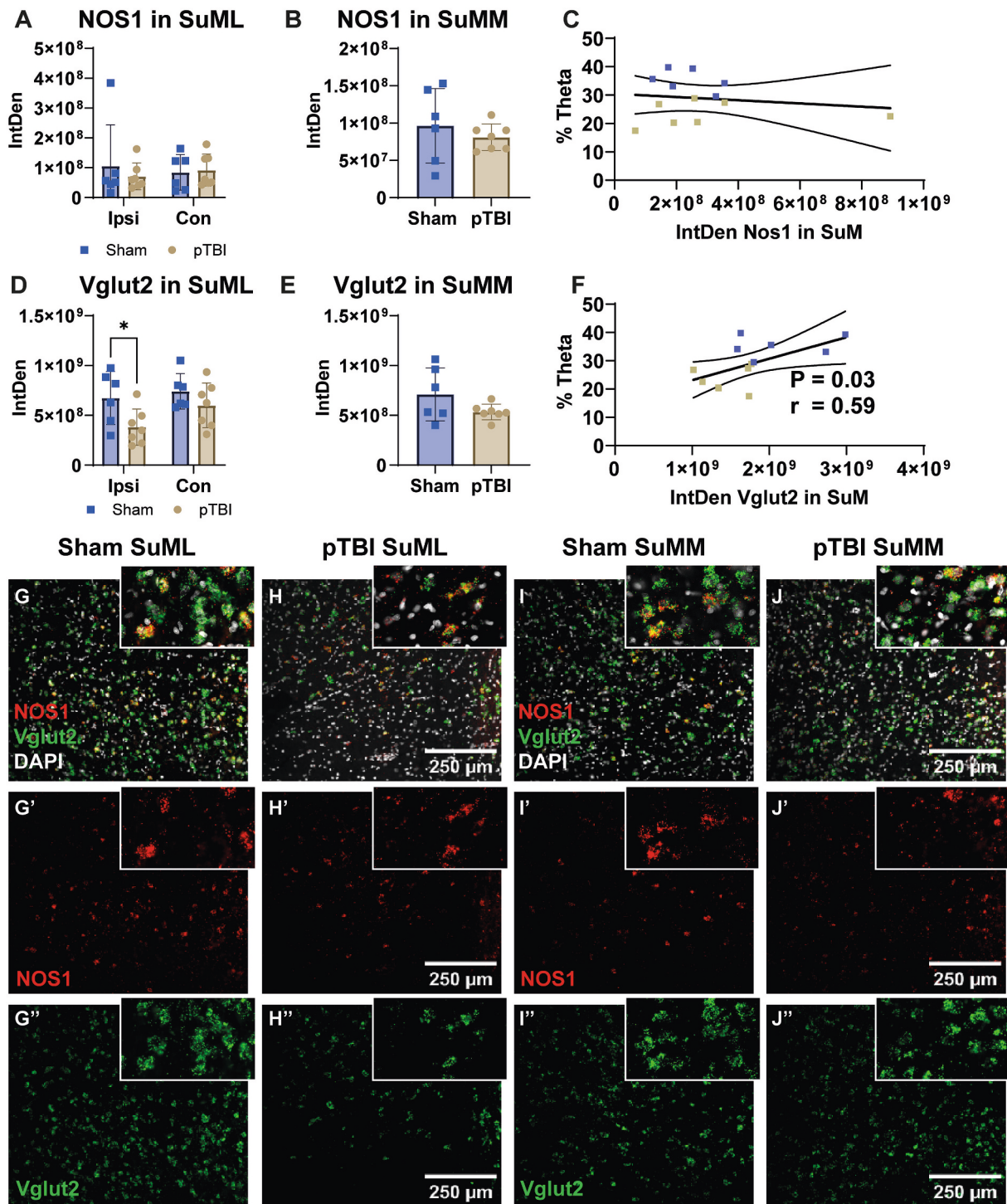


Fig. 6. NOS1 and Vglut2 transcript expression in the supramammillary nucleus following severe focal pTBI.

(A) NOS1 transcript levels were not altered in the SuML (Group, $F(1, 11) = 0.1213$, $p = 0.7342$; Hemisphere, $F(1, 11) = 4.301 \times 10^{-5}$, $p = 0.9949$; Hemisphere $\times$ Group, $F(1, 11) = 0.8620$, $p = 0.3731$).

(B) NOS1 transcript levels were not altered in the SuMM, Welch's t -test, $n = 6$ sham, $n = 7$ pTBI, $t(6.088) = 0.7215$, $p = 0.4973$.

(C) Correlation analysis showed no association between total NOS1 IntDen in SuM and relative theta power. Pearson correlation $r = -0.1591$, $p = 0.6035$, 95% confidence interval -0.6529 to 0.4295 and $n = 13$.

(D) Vglut2 transcript levels were significantly reduced in SuML following pTBI, (Group, $F(1, 11) = 5.167$, $p = 0.0441$; effect of Hemisphere, $F(1, 11) = 3.986$, $p = 0.0712$; Hemisphere $\times$ Group, $F(1, 11) = 1.127$, $p = 0.3111$).

(E) The reduction in SuMM did not reach statistical significance, Welch's t -test, $n = 6$ sham, $n = 7$ pTBI, $t(5.749) = 1.572$, $p = 0.1691$.

(F) Correlation analysis between total Vglut2 IntDen in SuM and relative theta power showed a positive correlation. Pearson correlation $r = 0.5895$, $p = 0.0340$, 95% confidence interval 0.05703 to 0.8609 and $n = 13$.

Statistical significance for Šidák-corrected post-hoc comparisons is indicated as $*p < 0.05$.

(G, H) Representative fluorescence images of SuML from sham and pTBI animals.

(I, J) Representative images of SuMM from sham and pTBI animals are shown. Higher-magnification insets are shown for all images.

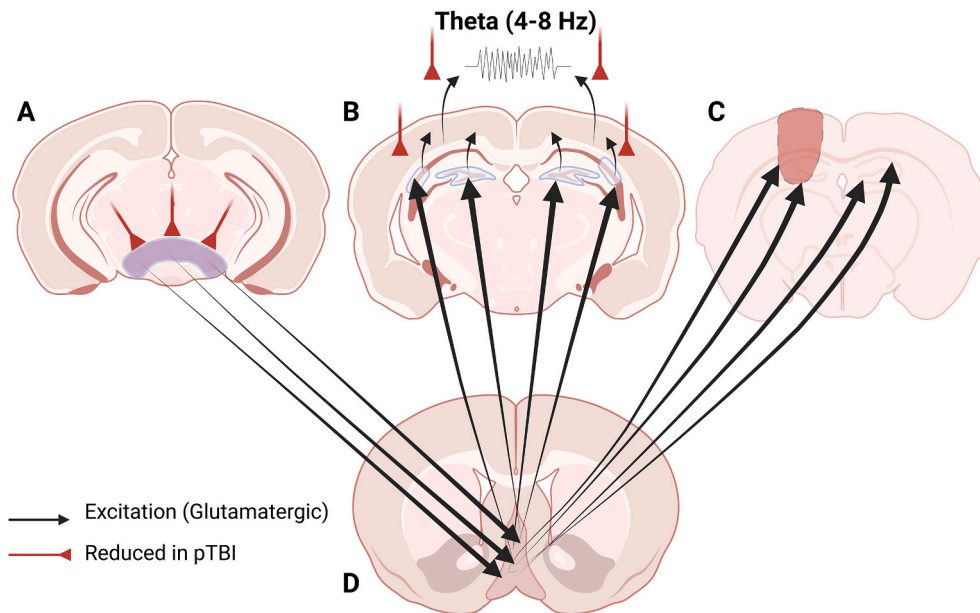


Fig. 7. Proposed model of REM-theta circuit disruption following pTBI. Schematic representation of the SuM-hippocampus circuit involved in generating and propagating REM-associated theta oscillations. Coronal schematics illustrate the anatomical levels analyzed and the proposed circuit interactions underlying REM-theta regulation.

(A) The SuM where Vglut2 transcript levels were reduced following pTBI.

(B) The dorsal hippocampus, including the CA2 region and DG, where reduced Vglut2 expression was observed following pTBI.

(C) The cortical and hippocampal level containing the focal injury site, illustrating the rostral position of the lesion relative to the analyzed dorsal hippocampus level.

(D) The medial septum relay linking SuM projections to the hippocampus. Black arrows indicate excitatory projections within the SuM-hippocampal circuit and ascending pathways supporting hippocampal and cortical theta activity during REM. Red symbols denote regions or networks proposed to be reduced following pTBI.

1992) through its excitatory projections to the medial septum (Borhegyi et al., 1998; Kesner et al., 2021) and hippocampus (Vertes et al., 2007; Verwer et al., 1997; Hashimoto et al., 2018; Vertes and McKenna, 2000), which in turn project to cortical regions and coordinate cortical oscillations during REM sleep (Renouard et al., 2015). Among the SuM subregions, SuML projects densely to the hippocampus whereas SuMM projections are sparse (Vertes, 1992). The hippocampus serves as a theta-generating source for the cortex (Buzsáki, 2002). The generation of theta activity in both the hippocampus and parts of the cortex is also dependent on a phasic input from the medial septum (Kirk, 1998). Several studies have identified the SuM as an important glutamatergic input to the DG and CA2 (Hashimoto et al., 2018; Billwiller et al., 2020), and glutamatergic Vglut2-expressing neurons in the SuM have been shown to contribute to generating REM-associated theta activity (Pedersen et al., 2017).

Given this well-established relationship between glutamatergic SuM-Hippocampal signaling and REM-theta oscillations (Buzsáki, 2002; Boyce et al., 2016), reduced Vglut2-mediated neurotransmission within the SuM-Hippocampal circuits may contribute to the attenuation of REM-theta activity following TBI.

The group effect of the Two-Way-ANOVA showed decreased Vglut2 levels in the SuML. Reduced Vglut2 transcript levels in the SuML support an altered glutamatergic signaling to the hippocampus. However, the hemisphere effect was only significant on the ipsilateral side, meaning that the observed effect on the SuML alone cannot fully explain the bilateral reduction in hippocampal Vglut2 levels. Reduced SuML Vglut2 may contribute to REM-theta suppression through ipsilaterally impaired SuM-septal modulation of hippocampal Vglut2, but the bilateral hippocampal Vglut2 reduction may additionally reflect altered SuM-derived terminals, like the SuM-fornix projections (Vertes, 1992), other glutamatergic afferents, axonal degeneration or broader synaptic remodeling after pTBI. Projection-specific tracing or circuit-selective manipulation will be required to resolve these mechanisms.

The effect of TBI on the SuM, which is remotely located relative to the site of impact, indicates long-range network effects of the injury. The etiology of this effect remains largely unclear but reported OXA projections from the LHA to the SuM (Peyron et al., 1998) offer a plausible contributing factor, bridging the observed reduction in OXA and Vglut2-positive neurons. In addition, it is worth noting that altered glutamatergic signaling, although not specifically within the SuM-hippocampal circuit, has previously been reported in multiple TBI models. For instance, post-TBI glutamatergic dysfunction has been observed in amygdala (Beitchman et al., 2019) and cortex (Dorsett et al., 2017). Further linking Vglut2-mediated neurotransmission to the REM sleep reduction, neurons responsible for initiating and maintaining REM sleep in the sublaterodorsal tegmental nucleus (also called locus subcoeruleus) express Vglut2 (Boissard et al., 2002; Clément et al., 2011).

The present injury model induces a large cavity and loss of brain tissue, mainly at the level of the rostral part of the ipsilateral dorsal hippocampus. Although parts of the ipsilateral cortex, fornix and hippocampal tissue are lost, the medial septum, which links the SuM to the hippocampus, is largely intact (Plantman et al., 2009) (Fig. 1K). Thus, the reduction in hippocampal Vglut2 is not solely explained by structural circuit disruption in the SuM-to-medial septum-to-hippocampus circuit. This claim is supported by the fact that even when normalizing hippocampal Vglut2 to NeuN, to adjust for any neuronal loss, TBI animals exhibited less hippocampal Vglut2 than sham animals. This is particularly important since we observed a modest loss of NeuN in the DG of pTBI animals compared to sham. Nevertheless, SuM is also known to project densely to several other structures which in turn project strongly to the hippocampus. Some examples are the nucleus reuniens of the thalamus, the medial and lateral septum, the entorhinal cortex, and the fornix (Vertes, 1992; Davidsson and Risling, 2015). Given that some of these structures are injured because of the injury site of the pTBI model, physical disruption cannot be fully excluded in explaining part of the observed reduction in theta oscillatory activity and hippocampal

Vglut2 levels. Most likely, a combination of these structural and circuit-level aspects is contributing to the suppression of the SuM-hippocampal circuit.

4.4. Spectral power changes beyond theta oscillations

It is suggested that TBI affects the microstructure of sleep more prominently than the macrostructure of sleep (Mantua et al., 2018). Human EEG studies have consistently reported increased delta and reduced alpha/theta power following TBI, reflecting large-scale disruption of cortical oscillations (Arakaki et al., 2018; Rapp et al., 2015; Lewine et al., 2019; Munia et al., 2017). This clinical pattern parallels the present findings, where spectral alterations were more widespread than changes in total sleep-state duration. In this context, REM-theta power may represent a clinically accessible EEG-derived marker of post-traumatic network dysfunction, linking sleep microstructure to underlying circuit-level pathology.

The concurrent reductions in REM sleep, theta power, and hypothalamic neuropeptide levels observed here mirror clinical findings of REM instability and cortical oscillations in patients with moderate to severe TBI, suggesting that the rodent model used in this study captures key aspects of human pathophysiology (Grima et al., 2016; Mathias and Alvaro, 2012). Comparable preclinical evidence is limited (Smith et al., 2021), but the present findings are in line with recent rodent EEG studies (Mountney et al., 2021; Thomasy and Opp, 2019; Korthas et al., 2022; Konduru et al., 2022; Paterno et al., 2016; Bottom-Tanzer et al., 2024).

Given its association with attention and working memory (Buzsáki, 2002), theta suppression may relate to observed memory impairments in this pTBI model (Plantman et al., 2012). This is supported by reports associating reduced theta oscillations with impaired cognitive and memory performance (Klimesch, 1999).

Although the focus of this study was to examine the effect of pTBI on SuM-hippocampal regulation of theta oscillations, we also found that other frequencies were altered and should be studied in more depth in future work. For instance, we observed a prominent increase in delta oscillations across all sleep-wake states, although delta power is usually low during REM sleep. Disrupted delta oscillations have been reported by others (Modarres et al., 2017), and increased delta oscillations are associated with increased daytime sleepiness (Arbour et al., 2015). Additionally, alpha oscillations, which are involved in attentional gating (Klimesch, 1999), were also suppressed. Attentional impairments after TBI are frequently reported (Cristofori and Levin, 2015), and the observed alpha suppression may reflect disrupted attention. However, it should be noted that we used standard definitions for each frequency band in this study, although it has been reported that the rat alpha band may overlap with a broader theta range, usually in the range 4–12 Hz (Jing et al., 2016; Corsi-Cabrera et al., 2001). This is important when interpreting the effects on theta and alpha, and it should be considered that the separately reported effects on theta (4–8 Hz) and alpha (8–12 Hz) may reflect broader changes in rat theta-related activity rather than two distinct frequency-band effects.

Moreover, convergent findings in humans and rodents have indicated diffuse, state-nonspecific cortical slowing following TBI (Modarres et al., 2017). In this study, we observed increased delta power with concomitant decreases in theta, alpha, and sigma power across sleep-wake states. Given these state-nonspecific changes, we acknowledge that a non-negligible component of the spectral findings reflects generalized post-traumatic slowing in addition to the proposed circuit-specific effect.

5. Limitations and future studies

First, translation of these findings to humans is limited by important species differences in sleep architecture. Rats are nocturnal night-active animals, meaning that the daytime-sleep in rats is more analogous to nighttime-sleep in humans. Additionally, they are polyphasic sleepers

sleeping during multiple periods across both day and night. This means that observed effects of TBI on rat sleep during the night, which is analogous to human daytime waking period, are more difficult to translate to humans than the effects observed during daytime.

Second, the link between TBI, SuM-hippocampal Vglut2 signaling, and REM-theta suppression remains associational. Future studies should investigate potential causal relationships.

Third, during the initial post-operative period animals in both the pTBI and sham groups were likely affected by anesthesia and post-surgical analgesia, which are known to reduce REM sleep, decrease theta power, and increase delta power (Wang and Teichtahl, 2007; Leszkowicz et al., 2007). This might partly explain the reduction in REM sleep and REM-theta power, accompanied by increased delta power, particularly during the first days and nights after surgery. However, the effects of anesthesia and analgesia alone cannot explain the persistent alterations in REM sleep and theta power that were observed across the two-week experimental period. Furthermore, the given effects of analgesia and anesthesia on REM would be the same for both Sham and pTBI groups, since both groups were treated identically, and the effect remained even when excluding the days and nights during which animals were given analgesia.

Fourth, the NOS1 analysis is limited by the fact that it did not specifically assess SuM neurons co-expressing NOS1 and Vglut2, nor the activity of these neurons. This should be explicitly studied in future work.

The relatively small number of biological replicates is a limitation, particularly for the correlation analyses. However, as previously discussed, this study examined within-animal-histology correlations rather than cross-cohort inference, which is common in experimental TBI studies, thereby aiming to examine novel aspects of sleep disturbances.

Although the two-week window enabled characterization of sub-acute dynamics, longer-term follow-ups are needed to assess whether oscillatory and neuropeptide changes persist in the chronic phase. The effects seen on both the SuM-hippocampal circuit and the LHA, including suppression of OXA and MCH, may be dependent on temporal changes and may differ in the chronic phases. These conclusions are thus limited to a two-week post-injury time window, and future studies should focus on analyzing chronic timepoints. Additionally, accumulating evidence indicates important sex-dependent differences in sleep regulation and TBI pathology (Leszkowicz et al., 2007). Future studies should include both sexes to determine whether these effects generalize across biological backgrounds.

In this study we used a focal TBI model with known behavioral sequelae and a well-characterized, anatomically distinct injury. To further elucidate the effects of TBI on the SuM-hippocampal circuitry, future studies should employ mild, diffuse TBI models. This would help determine whether there is a functional suppression in REM-associated theta oscillations in the absence of physical disruption of the SuM-hippocampal projections or tissue loss.

We also observed prominent alterations across multiple frequency bands. Although the present study focused on the REM-theta-generating circuit and the theta band, future work should investigate other frequency bands and their underlying mechanisms and functional implications. Finally, while our findings show strong associations and correlations between cortical oscillations and hypothalamic pathology, causal mechanisms remain to be established. Targeted manipulation of OXA, MCH, and SuM-hippocampal circuits will be essential to validate these pathways and evaluate their therapeutic potential. We are currently analyzing behavior and chronic effects of pTBI in an upcoming study.

6. Conclusion and translational significance

This study identifies an association between REM-theta-suppression and alterations in the SuM-hippocampal network following severe focal penetrating TBI. The concurrent suppression of REM sleep, attenuation

of theta oscillations, and reduction in Vglut2-related glutamatergic signaling within the SuM–hippocampal pathway support a circuit-specific mechanism underlying post-traumatic sleep dysfunction.

By demonstrating that REM-theta oscillations are associated with defined neuroanatomical alterations, these findings strengthen the translational bridge between experimental TBI models and human disease. EEG-derived spectral markers, particularly REM-associated theta power, may therefore represent clinically applicable biomarkers for monitoring network integrity, disease progression, and therapeutic response. Importantly, the circuit specificity observed here suggests that interventions targeting REM-regulatory systems, including OXA, MCH, or SuM–hippocampal pathways, may offer more mechanism-driven and potentially more effective strategies than nonspecific hypnotic approaches for improving outcomes in patients with TBI.

CRedit authorship contribution statement

Zabih Aurfan: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anette Fransson:** Supervision, Methodology. **Johan Davidsson:** Writing – review & editing, Supervision, Methodology. **Mårten Risling:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Mattias Günther:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Sebastian Thams:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Conceptualization.

Ethics declaration

Animal subject

This study was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

This study was approved by the Stockholms djuretiska nämnd. (Approval No. 3982020)

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors have no relevant affiliations, financial involvement, or conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2026.107612>.

Data availability

Data will be made available on request.

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