筑　波　大　学

博 士 （医 学） 学 位 論 文

Regulation of sleep by traumatic stress

（外傷性ストレスによる睡眠制御
メカニズムの解明）

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筑波大学大学院博士課程人間総合科学研究科

Lou Tingting

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Abstract

Sleep disturbances have been recognized as a core symptom of post-traumatic stress disorder (PTSD). However, the neural basis of PTSD-related sleep disturbances remains unclear. It has been challenging to establish the causality link between a specific brain region and traumatic stress-induced sleep abnormality. Here, we found that single prolonged stress (SPS) could induce acute changes in sleep/wake duration as well as short and long-term electroencephalogram (EEG) alterations in the isogenic mouse model. Moreover, the medial prefrontal cortex (mPFC) showed a persistently high number of c-fos expressing neurons, of which more than 95% are excitatory neurons, during and immediately after SPS. Chemogenetic inhibition of the prelimbic region of mPFC during SPS could specifically reverse the SPS-induced acute suppression of delta power (1-4Hz EEG) of non-rapid-eye-movement sleep (NREMS) as well as most long-term EEG abnormalities. These findings suggest a causality link between hyper-activation of mPFC neurons and traumatic stress-induced specific sleep-wake EEG disturbances.

Introduction

Post-traumatic stress disorder (PTSD)

Post-traumatic stress disorder (PTSD) is a more and more being emphasized diagnosis used to term individuals who suffer from neuropsychological symptoms following the experience of a psychologically disastrous traumatic event., such as domestic violence, rape, burglary, war, torture, and accidents (Breslau N,1988; Giora Pillar,1999). Exposure to catastrophic traumatic events could lead to PTSD-related symptoms characterized by intense fear, flashbacks, numbing, avoidance, hyperarousal, nightmares, and inability to cope with the demands of daily life (Breslau N,1988; Giora Pillar,1999; Germain, 2013; Nedelcovych et al., 2015; Deslauriers et al., 2018;). The dysregulation of emotion, such as fear, stress, and anxiety may lead to these symptoms of PTSD (Gros et al., 2011; Jovanovic and Ressler, 2010; Langeland and Olff, 2008; Nemeroff et al., 2006). This dysregulation of emotion may be influenced by a persistent, unusual adaptation of the neurobiological systems to the stress of the witnessed trauma (Sherin and Nemeroff, 2011). Although the exact aetiology of PTSD remains unknown, studies on PTSD neurobiology have revealed a myriad of neurotransmitter and neuroendocrine systems altered, including serotonin, catecholamine, amino acid, peptide, and opioid neurotransmitters, additionally dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Cohen et al., 2009; Simeon et al., 2007).

PTSD and sleep disturbances

Sleep disturbances are a core symptom of PTSD patients. PTSD patients usually complained about the difficulties in falling asleep, constant awakenings from sleep (with further problems falling back to sleep), shorter sleep period, restless sleep, daytime fatigue, and particularly nightmares and anxiety dreams (Neylan TC,1998; Mellman TA,1995). Additionally, other symptoms have also been reported, such as increased rapid eye movement sleep (REMS) phasic EMG activity, increased startle response and possibly raised the awakening threshold from sleep (Giora Pillar, 2000).

There is potent evidence for that these patients are hyperarousal and have a complaint of difficulties being asleep, but once they fall asleep, it will be deeper. If the “sleep deepening” mechanism is dominant, it may result in an increased percentage of slow-wave sleep. However, if the “hyperarousal” dominates, the final result will be opposite, insomnia (Giora Pillar, 2000). Moreover, polysomnographic studies conducted in PTSD patients reported abnormal sleep architecture (Kobayashi et al., 2007; Yetkin et al., 2010) and sleep-disordered breathing (Yesavage et al., 2012), revealing deficits in both REM and non-rapid-eye movement (NREM) sleep, including reduced and fragmented NREM and REM sleep, shortened latency to REM sleep, and increased REM density. The nightmares and anxiety dreams correlated with PTSD may be comparatively unique for this disorder, and the mechanisms of dysfunctional REM sleep may be engaged in the pathogenesis of the post-traumatic anxiety dream (Mellman TA, 1995. Ross RJ, 1994. Van der Kolk B, 1984). The hypothesis which is proposed by Ross and colleagues in 1989, that REMS disturbances are the characteristic of PTSD, this hypothesis has prompted plenty of clinical, preclinical, and animal studies on the role of sleep in the pathophysiology of PTSD. The alterations of REM sleep may be reduced by other factors, suchpsychiatric comorbidity, history of addictive disorders and treatment (Kobayashi, 2007). However, previous findings regarding traumatic stress-induced sleep abnormalities have not always been consistent, such as changes in sleep amount, sleep latency and number of nightly awakening (S. Yetkin, 2010; I. Kobayashi, 2007)

Medial prefrontal cortex (mPFC) is a top-down control center for stress response regulation

Importantly for PTSD, the prefrontal cortex (PFC) plays a critical role in the regulation of fear learning, expression, and extinction (Gilmartin MR,2014). The function of two PFC subregions, the anterior cingulate cortex (ACC) and the medial PFC (mPFC) has been found to be altered in PTSD (Pitman RK,2012; Garfinkel SN,2009). Numbers of regions in the central nervous system (CNS) show increased immediate–early gene expression after stressor exposure, including mPFC (Cullinan

et al., 1995; Ostrander et al., 2003), suggesting that mPFC should be associated in stress response regulation. Indeed, the mPFC is one of the most important top-down control centres that regulate the overall stress response (Diorio et al., 1993; Figueiredo et al., 2003; McDougall et al., 2004; Radley et al., 2006). Moreover, mPFC is a necessary neural substrate for consolidating cognitive-affective information and controlling the HPA axis response to emotional stress. Emotional stressors are regarded as stimuli involved in distinct cognitive and affective components. This type of stressors could active a series of cell groups in the limbic forebrain, including aspects of the septum, amygdala, bed nucleus, hippocampus, and mPFC (Cullinan et al., 1995; Campeau et al., 1997; Li and Sawchenko, 1998; Dayas et al., 2001). These brain regions are suggested that they are regulated in positive or negative modulation of the HPA by neuronal mechanisms and as targets of glucocorticoid negative feedback (Sapolsky et al., 1984; Kovacs and Makara, 1988; Herman et al., 2003, 2005). Moreover, from the previous study, mPFC is involved in the regulation of neuroendocrine, autonomic and behavioural aspects of stress responses (Jinks & McGregor, 1997; Buijs & Van Eden, 2000; Van Eden & Buijs, 2000; Sullivan & Gratton, 2002). The prelimbic (PrL) region of mPFC is a major subregion that controls neuroendocrine outputs of the paraventricular hypothalamic nucleus (PVH) to restore homeostasis of the HPA axis-the central stress response system (Radley et al., 2006; Herman et al., 2012), as has been indicated in response to some (but not all) physical and psychological or emotional experimental stressor paradigms (Sullivan & Gratton, 1999; Crane et al., 2003; Figueiredo et al., 2003).

Electroencephalogram(EEG)

Electroencephalography (EEG) has been instrumental in making modern sleep research becoming possible. By placing electrodes on the scalp, the EEG records the voltage changes within the thousands to millions of neurons in the cerebral cortex. EEG could be a much more powerful and meaningful brain measurement tool, we could use it to identify one-to-one mappings between EEG feature and neural microcircuit configuration. The signals of EEG were divided into three stages,

wake(low voltage, high-frequency irregular EEG pattern, with a high-level EMG activity), NREM sleep (presence of slow waves, high amplitude and low-frequency EEG pattern, with a low level of EMG activity), and or REM sleep (low voltage, higher frequency EEG dominated by theta-activity in the occipital derivation, with a low level of EMG activity). Typically, Power spectral analysis of EEG was used to quantify the frequency content into four distinct bands, delta (1–4 Hz), theta (5–8 Hz), alpha (9–14 Hz) and beta (15–30 Hz).

There are also inconsistent results from quantitative analysis of the sleep-wake EEG of PTSD patients (Campbell, 2009). For example, there have been reports of increased (Woodward et al., 2000; Insana et al., 2012), decreased (Cohen et al., 2013), or no difference (Mellman et al., 2007) in the beta power of EEG during REMS in adult PTSD patients. Both reduced and increased delta power activity during NREMS and REMS have also been reported in PTSD patients (Woodward et al., 2000; Germain et al., 2006; Insana et al., 2012). These conflicting findings may be attributed to the inter-individual differences and disease heterogeneity, such as differences in initial traumatic stimuli, analysis stages of the illness, comorbidities with other psychiatric conditions, and the diversity of underlying neural mechanisms (Kobayashi et al., 2007; Yetkin et al., 2010; Germain, 2013; Baglioni et al., 2016; Khazaie et al., 2016). In addition, EEG studies have shown mPFC contributes to the unique, comfortable sensation forming a diffuse alpha rhythm (Shin Yamamoto, 2006).

Single prolonged stress (SPS) paradigm

The single prolonged stress (SPS) paradigm is a pre-clinical model that recapitulates many of the behavioural, molecular and physiological alterations observed in PTSD patients, such as enhanced reactivity to trauma cues, impairment of fear extinction and extinction retention, hyperarousal, changes in neurotransmitter and neuropeptide systems, and altered responsivity of the HPA axis (Liberzon et al., 1997; Liberzon et al., 1999; Deslauriers et al., 2018). Since its initial description more than 20 years ago (Liberzon et al., 1997), the SPS procedure has been referred to by over 200 peer-reviewed studies. Although it is called a “single” prolonged stress, the procedure

includes physiological stress, psychological stress and endocrine stress. The prolonged stress begins with a 2-h restraint period that is immediately followed by a forced-swimming experience, lasting 15-min, and recovering 15-min, then a brief loss of consciousness induced by ether exposure. After recovery, mice remain undisturbed for 7-day (Liberzon et al., 1997). Previously, the effects of SPS on the sleep-wake architecture have only been investigated in two rat studies that yielded inconsistent results (Nedelcovych et al., 2015; Vanderheyden et al., 2015). While one study showed that SPS caused an increase of REMS in the dark phase, but no change in NREMS (Vanderheyden et al., 2015), another reported that SPS reduced both NREMS and REMS in the light phase, followed by a strong rebound in NREMS and REMS in the dark phase (Nedelcovych et al., 2015). These inconsistent results across different laboratories need to be carefully re-examined (Deslauriers et al., 2018).

Animal model and clinical studies of PTSD have revealed structural and functional alterations in multiple brain regions. However, the neurological correlates of traumatic stress-induced sleep abnormalities remainmostly unexplored (Karl et al., 2006; Deslauriers et al., 2018; Mysliwiec et al., 2018). In particular, it has been challenging to establish the causality link between any specific brain region and traumatic stress-induced sleep abnormalities. Therefore, we also try to explore the neurobiological correlates of traumatic stress-induced sleep abnormalities in this study. We found that SPS-treated mice exhibited specific changes in the sleep-wake architecture, including both short- and long-term EEG alterations. Moreover, our results suggest for the first time a causality link between the hyper-activation of mPFC neurons and the SPS-induced specific sleep-wake EEG abnormalities. This type of investigations should be essential for understanding the neural mechanisms and facilitating the development of effective therapies for a subset of PTSD patients.

Objectives

First, we discovered single prolonged stress (SPS) could induce acute changes in sleep/wake duration and electroencephalogram (EEG) power spectrum, as well as long-term EEG alterations. These can reflect the change of post-traumatic stress disorders (PTSD) patients. However, the neural basis of PTSD-related sleep disturbances remains unknown. Then we found that the medial prefrontal cortex (mPFC) showed persistent activity during and after traumatic stress. Finally, we wished to verify the causality link between the specific brain region and traumatic stress-induced sleep abnormality.

Hypothesis

Traumatic stress induces acute changes in sleep/wake duration, EEG power spectrum and long-term sleep/wake EEG abnormalities.

Chemogenetic inhibition of mPFC, specifically reverses SPS-induced seep/wake EEG disturbances.

Materials and Methods

Animal Subjects.

All mice were housed under humidity and temperature (22–25± ℃) controlled conditions on a 12-h light–dark cycle with food and water provided ad libitum. We used 12–20 weeks old (26-33 g body weight) C57BL/6N male mice (CLEA Japan) in this study. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba and thus were in accordance with NIH guidelines. All mice were singly housed for one week before each experiment.

Sleep Deprivation and Single Prolonged Stress.

For sleep deprivation, mice were sleep deprived for 4 h from the onset of the light

phase (ZT0-ZT4) by gently touching the cages when they started to recline and lower their heads in the home cage. The single prolonged stress (SPS) was performed at the onset of the light phase (ZT0) as previously described (Liberzon et al., 1997; Liberzon et al., 1999; Deslauriers et al., 2018). First, each mouse was restrained for 2 h in a 50 ml 1 Falcon tube with the bottom removed. Second, the mouse was forced to swim for 20 min in a plastic cylinder (height: 25 cm; diameter: 18.5 cm) filled with water (21-24 °C), such that the mouse's hind limbs could not touch the bottom. Third, after recuperating for 15 min in a new cage, the mouse was exposed to ether until general anesthesia (no more than 5 min). Finally, the mouse was returned to its home cage (around ZT3.5) and sleep-deprived until ZT4 by gently touching the cages.

EEG/EMG Electrode Implantation

Mice (8–10 weeks old) were implanted with the EEG/EMG electrodes under anesthesia by isoflurane (3% for induction, 1% for maintenance). Briefly, four electrode pins were lowered to the dura under stereotaxic control. Two electrodes for EEG signals were positioned over the frontal and occipital cortices [anteroposterior (AP): 0.5 mm, mediolateral (ML): 1.3 mm, dorsoventral (DV): −1.3 mm; and AP: −4.5 mm, ML: 1.3 mm, DV: −1.3 mm]. Two electrodes with flexible wires for EMG recording were threaded through the dorsal neck muscle. Afterwards, the EEG/EMG electrodes were glued to the skull with dental cement. Mice were individually housed following surgery and allowed a minimum recovery period of seven days.

Sleep-wake Behaviors Analysis

The sleep–wake behaviours were analyzed as previously described (Funato et al., 2016; Wang et al., 2018). Mice were tethered to a counterbalanced arm (Instech Laboratories) that allowed free movement and exerted minimal weight, and acclimatized to the recording chamber around seven days before recording. EEG/EMG signals were recorded at the age of 12–20 weeks; age-matched animals were used in each experiment for control and treatment groups. EEG/EMG data were

analyzed using a MatLab (MathWorks)-based 1 semi-automated staging software followed by manual correction. EEG signals were decomposed by fast Fourier transform analysis for 1 to 30 Hz with 1 Hz bins. Sleep/wake states were scored in 20 s epoch as wake (low amplitude, fast EEG and high amplitude, variable EMG), REMS (dominant theta (5-8 Hz) EEG and EMG atonia), or NREMS (high amplitude delta (1-4 Hz) EEG and low EMG tonus). Absolute (arbitrary units) and relative EEG power density analysis was performed to examine the delta (1–4 Hz), theta (5–8 Hz), alpha (9–14 Hz) and beta (15–30 Hz) activities during NREMS, REMS, or wake state at indicated ZT period. To minimize the inter-individual differences for following statistical analysis, absolute EEG power data of each individual animal for the corresponding NREMS, REMS, or wake state was normalized to the mean power from ZT8 to ZT11 of baseline recording day of all animals used within each corresponding experiment, which is at the end of the major rest period (Franken et al., 2001; Mang et al., 2016). Relative EEG power density analysis (%) is defined by the ratio of a specific frequency bin to the total power over all frequency bins (1–30 Hz). In hourly analysis of sleep-wake architecture, each data point represents the mean value of either duration or EEG power density in the following 1 h during NREMS, REMS, and wake states. Researchers were blinded to genotype and/or treatment before data analysis, and only animals with unreadable EEG signals were excluded from final analysis.

Behavioral Experiments

Two groups of mice were sleep deprived for 4 hours (SD4) or exposed to SPS treatment, respectively. On the seventh day after the SD4/SPS procedure, the tail suspension test (TST) was performed as previously described (Can et al., 2012b). Each mouse was suspended in the hook of an open front tail suspension test box, approximately 50 cm above the surface of table with a small piece of adhesive tape placed 2 cm away from the tip of the tail. The duration of immobility was recorded for 10 minutes by a video camera positioned in front of the test box. Mice were

considered immobile only when they hung passively and were completely motionless. Mice were returned to their home cages to rest for at least 1 hour, and then the forced swim test (FST) was performed as previously described (Can et al., 2012a). The mice were placed individually for 10 minutes in a plastic cylinder (height: 25 cm; diameter: 18.5 cm) filled with water (21-24 ℃) to a depth of 14 cm. The water depth was adjusted so that the animal's hind limbs cannot touch the bottom. Water was changed between subjects. All test sessions were recorded by a video camera positioned on the top of the plastic cylinder. Mice were considered to be immobile when floating motionless or making only those movements necessary to keep its head above the water. The duration of immobility was measured manually by an observer blind to group assignment.

Immunohistochemistry

All mice were singly housed at least for one week before experiments. After experimental treatments, test mice were allowed to recover in the home cage. Specifically, at least one paired control and stressed mice brains were harvested at 30 min (ZT4.5) or 3.5 h (ZT7.5) after SPS or SD4 treatment at the same experimental day and processed at the same time in the following steps. At indicated ZT time, paired control and stressed mice were rapidly anesthetized with pentobarbital (50 mg/kg, i.p.), and then transcardially perfused with 0.1 M phosphate buffer saline, pH7.4 (PBS), followed by 4% paraformaldehyde in PBS (PFA). Whole brain was dissected and post-fixed for 24-h in 4% PFA at 4 ℃, and then cryoprotected with 30% sucrose (wt/vol) in PBS for 48 h at 4 ℃. The tissues were frozen in the Tissue-Tek O.C.T compound (Sakura Finetek), and 80-μm-thick coronal sections were cut on a cryostat (CM3050S, Leica). For c-Fos staining, the floating brain sections were washed three times with PBS for 5 min each, incubated with 1% Triton X-100 in PBS for 2 hours. The sections were incubated in 10% Blocking One (nacalai tesque) in PBS with 0.1% Triton-X-100 (blocking solution) for 1-h at room temperature. The sections were incubated with rabbit anti-c-Fos antibody (1:2500, EMD Millipore,

ABE457) in blocking solution at 4 ℃ overnight. After washing three times with PBS, the sections were incubated with Donkey anti-rabbit Alex488 (1:500, Thermo Fisher R37118) and Fluorescent Nissl Stain (1:500, Thermo Fisher N21479) in Blocking solution at 4 ℃ overnight. After washing three times with PBS, the sections were mounted and covered with coverslip. All images were acquired using the Zeiss LSM700 confocal microscope with a 10×12 objective lens (NA = 0.45) under the Zen 2010 software (Carl Zeiss). The c-Fos positive neurons were counted in all sections from the same mouse brain (Image J). No normalization was performed for the c-fos expression at ZT4.5 and ZT7.5. Representative images shown in the figures were chosen from a similar region based on morphology.

In Situ Hybridization

The cDNA fragments of mouse c-fos, vGlut1, and vGat were amplified by PCR with antisense primers containing T3 or T7 promoter sequence. In vitro transcription was performed with PCR-amplified template using T3 RNA polymerase (Promega) or T7 RNA polymerase (Roche) for the synthesis of antisense probes. Fluorescent two-color in situ hybridization was performed based on a basic method (Ishii et al., 2017). Briefly, mice were subjected to SPS treatment and, after 30 min, were anesthetized with pentobarbital (50 mg/kg, i.p.) followed by perfusion with 4 % paraformaldehyde (PFA) in PBS. 1 Brain slices (40 μm) were treated with protease K (Roche, cat#03115887001), followed by acetylation. The brain slices were incubated with hybridization buffer containing RNA probe mix at 60 ℃ for 16 hours. After stringent washing, brain slices were incubated with horseradish peroxidase (HRP) conjugated anti-FITC antibody (PerkinElmer, 1:1000) or HRP-conjugated anti-Dig antibody (Roche; 1:1000) overnight at 4 ℃. TSA system (TSA-FITC or TSA-Biotin; PerkinElmer) was applied to visualize the mRNA signal. All images were acquired using the Zeiss LSM700 confocal microscope with a 10× objective lens (NA = 0.45) under the Zen 2010 software (Carl Zeiss). The c-fos, vGlut1 and vGat positive neurons were counted in all sections from the same mouse brain (Image J).

Stereotaxic AAV Injection and Drug Administration

For bilateral injection of AAV (AAV2/9-CMV-mCherry; AAV2/9-hSyn-hM4Di–mCherry) into the medial prefrontal cortex, male mice (8–10 weeks old) were anaesthetized with isoflurane (3% for induction, 1% for maintenance) and placed in a stereotaxic frame (David Kopf Instruments). An incision was made on the top of the skull, and the skin was retracted and connective tissue gently scraped away. After exposing the skull and cleaning the surface with 3 % hydrogen peroxide, bilateral craniotomies (~1 mm diameter each) were made to allow virus delivery (500 nl at 100 nl/min). Stereotaxic coordinates of virus injection were based on Paxinos and Franklin mouse brain atlas (AP: −1.94 mm, L: ±0.4mm, DV: −2 mm). For EEG/EMG analysis of AAV-injected mice, the EEG/EMG electrode implantation was performed immediately following AAV injection. Clozapine N-oxide (CNO; Cayman Chemical, Item No.12059) was dissolved in saline. Vehicle (0.9% saline) or CNO (3 mg/kg) was administered by intraperitoneal injection at ZT0 and ZT3.5 before the mouse returned to the home cage.

Statistical Methods

GraphPad Prism 6 software was used for statistical tests. No statistical method was used to predetermine sample size. Randomization was not used. Following two-way ANOVA analysis of variance, Sidak's test was performed to compare a set of means, repeated measures was performed for matched subject comparisons. Paired t-test was performed for matched subject comparisons, whereas unpaired t-test for group comparisons. The complete sample size, statistical test method and results for each comparison are reported in the figure legends and described in detail in Supplementary Table 1. $P<0.05$ was considered statistically significant. Unless otherwise noted, all experimental subjects are biological replicates and at least two independent experiments were performed.

Results

We adopted the standardized SPS paradigm to investigate the effects of traumatic stress on the sleep-wake architecture in wild-type C57BL/6N male mice. We used a longitudinal experimental design by sequentially comparing sleep/wake changes before and after 4-h sleep deprivation by gentle handling (SD4, ZT0-ZT4) or single prolonged stress (SPS, ZT0-ZT4) on the same subjects (**Figure 1)**. For the SD4 segment, after continuous 24-h baseline (SD4-BL) recording, all test mice are subjected to SD4 (SD4-D1) and continuously monitored for EEG and electromyogram (EMG) in the home cage until the seventh day (SD4-D7). After one to three days' rest, the same mice would be subjected to SPS (ZT0-ZT4) to study how traumatic stress caused sleep-wake disturbances. For the SPS segment, after continuous 24 h baseline (SPS-BL) recording, all test mice were subjected sequentially to 2-h restraining, 20-min forced swimming, and up to 5-min anesthesia by ether (SPS-D1), and 1 followed by continuous EEG/EMG recording until the seventh day (SPS-D7). This longitude design gave us two important advantages over previous studies: a) comparison of SPS and SD4 could distinguish the specific effects of SPS (as opposed to prolonged wakefulness) on the sleep-wake architecture; b) the baseline and post-SD4 or post-SPS EEG/EMG recordings of the same mice allowed for both absolute and relative EEG power analysis to comprehensively evaluate the SPS-induced short-term (D1) and long-term (D7) EEG abnormalities, which is not possible in previous SPS rat studies (Nedelcovych et al., 2015; Vanderheyden et al., 2015).

Traumatic Stress Induces Acute Changes in Sleep/Wake Duration

To examine the acute effect of traumatic stress on the sleep-wake architecture, we compared the EEG/EMG data of test mice on the day before (SD4-BL or SPS-BL) and after SD4/SPS (SD4-D1 or SPS-D1) (**Figure 2**). It is important to note that there were essentially no difference in the baseline sleep-wake pattern of the same mice before SD4 and SPS treatment (SD4-BL vs. SPS-BL), making it possible to directly compare the effects of SPS and SD4 on the sleep-wake architecture (**Figure 2A, Supplementary Figure 1A and Supplementary Table 1**). On the day after SD4,

there was on average a 58.1% reduction in REMS duration (SD4-D1, 2.6±1.9 min vs. SD4-D0, 6.2±1.9 min) at the first hour (ZT4) after sleep deprivation, and a 72.3% and 8.2% rebound of REMS (SD4-D1, 25.5±7.8 min vs. SD4-D0, 14.8±6.2 min) and NREMS (SD4-D1, 254.2±53.6 min vs. SD4-D0, 234.9±50.6 min) in the dark phase, respectively **(Figure 2A and Supplementary Figure 1B).** On the day after SPS, there was on average a 96.6% and 47.5% reduction in REMS duration (SPS-D1, 0.2±0.6 min vs. SPS-BL, 5.9±1.5 min) and NREMS duration (SPS-D1, 21.5±11.2 min vs. SPS-BL, 41.0±6.7min) at the first hour (ZT4) after SPS, as well as a 183.9% and 33.9% rebound of REMS (SPS-D1, 44.0±10.1min vs. SPS-BL, 15.5±7.3 min) and NREMS (SPS-D1, 325.4±49.5 min vs. SPS-BL, 243.1±37.6 min) in the dark phase, respectively **(Figure 2A and Supplementary Figure 1C)**.

By direct comparison of the SPS-D1 vs. SD4-D1 data, we found that SPS, relative to SD4, resulted in about 36.2% less NREMS at ZT4 (SPS-D1, 21.5±11.2 min vs. SD4-D1, 33.7±10.9 min) and 56.3% less REMS during ZT4-6 (SPS-D1, 6.3±3.1 min vs. SD4-D1, 14.4±4.0 min) **(Figure 2A)**. In the dark phase, SPS mice spent 28% and 72.5% more time than SD4 mice in NREMS (SPS-D1, 325.4±49.5 min vs. SD4-D1, 254.2±53.6 min) and REMS (SPS-D1, 44.0±10.1 min vs. SD4-D1, 25.5±7.8 min), respectively **(Figure 2B)**. Thus, our results indicate that traumatic stress by SPS can induce specific changes in the sleep-wake architecture that are distinct from sleep deprivation.

Traumatic Stress Induces Short-term Sleep/Wake EEG Abnormalities

Similarly, the baseline sleep/wake EEG power spectrum of the test mice was essentially the same before SD4 and SPS treatment (**Supplementary Figure 2**; SD4-BL vs. SPS-BL). By absolute EEG power analysis, SD4 resulted in a broad increase over baseline in all frequency bands of EEG signals during NREMS in the light phase, particularly in the first hour (ZT4) after sleep deprivation (↑56.4% delta; ↑24.3% theta; ↑15.1% alpha; ↑19.0% beta) (**Supplementary Figure 3;** SD4-D1 vs. SD4-BL). On the other hand, SPS resulted in a 13.2% and 9.5% increase over baseline, respectively, in the delta and theta power of EEG signals during NREMS at

ZT4 (**Supplementary Figure 3A**; SPS-D1 vs. SPS-BL). Comparison of the SPS and SD4 data reveals that SPS, relative to SD4, caused a broad suppression in all frequency bands of EEG signals during NREMS at ZT4 (↓26.1% delta; ↓12.1% theta; ↓18.0% alpha; ↓16.2% beta) and in the dark phase (↓9.0% delta; ↓11.0% theta; ↓7.2% alpha; ↓9.4% beta), as well as a specific suppression (↓9.4%) of NREMS delta power, a measurable index of sleep need, in the light phase (**Figure 3A-D**; SPS-D1 vs. SD4-D1), which may indicate the decreases of sleep propensity.

During REMS, SPS, relative to SD4, causes a significant increase in the absolute delta (↑8.8%), alpha (↑20.6%) and beta (↑10.6%) EEG power in the light phase, as well as a 10% reduction in theta EEG power in the dark phase (**Figure 3E-H**; SPS-D1 vs. SD4-D1). During wakefulness, SPS, relative to SD4, caused a significant decrease in absolute delta (↓16.3%) and beta (↓7.9%) power in the light phase (**Figure 3I-L**; SPS-D1 vs. SD4-D1). Additionally, SPS mice exhibited a broad reduction in all frequency bands of EEG signals (↓8.1% delta; ↓7.5% theta; ↓12.8% alpha; ↓12.6% beta) in the dark phase (**Figure 3I-L**; SPS-D1 vs. SD4-D1). These observations indicate that SPS causes specific short-term sleep/wake EEG abnormalities.

Traumatic Stress Induces Long-term Sleep/Wake EEG Abnormalities

To examine the long-term effect of SPS on sleep-wake architecture, we compared the EEG/EMG data of the same mice on the seventh day (D7) after SD4 and SPS treatment (**Figure 4A**; SD4-D7 vs. SPS-D7). Consistent with the previous study of SPS rats (Nedelcovych et al., 2015), there was no significant difference in the total duration, episode duration, or episode number of NREMS, REMS and wakefulness on D7 after SPS (**Supplementary Figure 4A-D**). By contrast, SPS, relative to SD4, caused a broad reduction in sleep/wake EEG power densities in the light phase, including the alpha (↓5.3%) and beta (↓6.4%) power during NREMS; the theta (↓4.9%) and alpha (↓4.8%) power during REMS; the alpha (↓3.5%) and beta (↓3.1%) power during wakefulness (**Figure 4B-D;** SPS-D7 vs. SD4-D7). In the dark phase, SPS caused a specific decrease in absolute theta (↓4.1%), alpha (↓6.7%) and beta (↓8.0%) power during NREMS; theta (↓7.8%) power during REMS; and alpha

(↓5.7%) power during wakefulness (**Figure 4B-D;** SPS-D7 vs. SD4-D7). Taken together, these observations indicate that unlike sleep deprivation, traumatic stress by SPS can lead to long-term sleep/wake EEG abnormalities.

Absolute EEG Power Analysis Is Superior to Relative EEG Power Analysis

Our previous studies suggest that relative EEG power analysis is likely to miss critical changes of the EEG signals, such as a global reduction in EEG power densities, which can be detected by absolute EEG power analysis (Funato et al., 2016; Wang et al., 2018). Accordingly, we obtained very different outcomes by relative EEG power analysis (**Figure 4E**). During NREMS, SPS, relative to SD4, causes variable changes in the EEG power spectrum in the light phase (delta,↓3.4%; theta,↑3.8%; alpha, ↑4.5%; beta,↑5.5%) and in the dark phase (delta, ns; theta, ↓1.8%; alpha, ↑2.4%; beta, ns). Remarkably, only the modest reduction in NREMS delta (↓3.4%) power is verified by absolute EEG power analysis (**Figure 4E**; SPS-D1 vs. SD4-D1). During REMS, relative EEG power analysis also reveals variable changes of the EEG power spectrum in the light phase (delta, ns; theta, ↓5.8%; alpha, ↑11.1%; beta, ns) and in the dark phase (delta, ns; theta, ↓4.2%; alpha, ns; beta, ns). Among these changes, only the 11.1% alpha power increase in the light phase and 4.2% theta power reduction in the dark phase are consistent with absolute EEG power analysis (**Figure 4E**; SPS-D1 vs. SD4-D1).

For the long-term sleep/wake EEG abnormalities, relative EEG power analysis reveals that SPS, relative to SD4, causes a modest reduction in the alpha (↓2.5%) and beta (↓4.0%) power of NREMS in the light phase, beta (↓4.1%) power of NREMS in the dark phase, and alpha (↓3.4%) power of wake in the dark phase. Although these changes are largely consistent with those of absolute EEG power analysis, relative EEG power analysis failed to detect many critical changes of the EEG power spectrum in the dark phase (**Figure 4E**; SPS-D7 vs. SD4-D7). Based on these observations, we conclude that absolute EEG power analysis is superior to relative EEG power analysis, which should be adopted especially in the longitude experimental setting.

Persistent Activation of mPFC Neurons During and After SPS Treatment

Accumulating studies suggest that PTSD may be mediated by structural and functional alterations in multiple brain regions, including the prefrontal cortex, locus coeruleus, amygdala, hippocampus, and the hypothalamic−pituitary−adrenal (HPA) axis (Lindauer et al., 2004; Wignall et al., 2004; Lindauer et al., 2006; Chen et al., 2018; Deslauriers et al., 2018; Logue et al., 2018; Naegeli et al., 2018; van Rooij et al., 2018; Heyn et al., 2019). To explore the neurobiological correlates of traumatic stress-induced sleep abnormalities, we performed comparative analysis of the expression of immediate early gene c-Fos by immunostaining of mouse brain samples harvested at ZT4.5 and ZT7.5 after SD4/SPS treatment (ZT0-ZT4) (**Figure 5A**). Because c-Fos proteins exhibit a half-life of 45 min for fast decay and 1.5–2 hours for slow decay (Shah and Tyagi, 2013), we wanted to identify specific brain regions showing persistent hyper-activity in response to SPS.

Although sleep deprivation or extended wakefulness can significant change the neuronal properties or c-fos expression of many different brain regions(Winters et al., 2011; Ren et al., 2018), we found at ZT4.5, SPS mice, relative to SD4 mice, showed significantly more c-Fos-expressing neurons in multiple subregions of the prefrontal cortex (**Figure 5B-D**), including the primary (M1) and secondary (M2) cortex of motor cortex (MC), the cingulate (Cg1), prelimbic (PrL), infralimbic (IL) and dorsal peduncular (DP) cortex within the medial prefrontal cortex (mPFC), and the medial (MO), ventral (VO), and lateral (LO) part of the orbitofrontal cortex (OFC) (**Figure 5D**). By two-color fluoresence in situ hybridization, we showed that more than 95% of c-fos positive neurons in the mPFC express the excitatory neuron marker vGlut1, but not the inhibitory neuron marker vGat (**Figure 5E, F**). Moreover, while SD4-induced c-Fos expression dissipated, SPS-induced c-Fos expression could still be observed in the medial prefrontal cortex (mPFC), most notably in the PrL, IL and DP at ZT7.5 (**Figure 5C, D**). These results suggest SPS causes persistent hyper-activities of mPFC neurons during and immediately after SPS treatment.

Chemogenetic Inhibition of mPFC Reverses SPS-induced Sleep/Wake EEG Disturbances

We hypothesized that the persistent hyper-activities of mPFC could contribute to the SPS-induced short- and long-term alterations in the sleep-wake architecture and EEG power spectrum. The prelimbic (PrL) region of mPFC is a major subregion to control neuroendocrine outputs of the paraventricular hypothalamic nucleus (PVH) to restore homeostasis of the HPA axis-the central stress response system (Radley et al., 2006; Herman et al., 2012). Therefore, we used the inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) system to investigate whether hyper-activation of PrL neurons play an important role in traumatic stress-induced sleep-wake disturbances. Specifically, we bilaterally injected Adeno-associated viruses (AAV) expressing mCherry (AAV2/9-CMV-mCherry) or hM4Di (AAV2/9-hSyn-hM4Di-mCherry) into the PrL of mPFC in C57BL/6N mice (**Figure 6A, B**). All AAV-injected mice were sequentially subjected to SD4 and SPS treatments as described above (**Figure 1**), except for intraperitoneal injection of vehicle during SD4 or CNO during SPS at ZT0 and ZT3.5, and followed by continuous EEG/EMG recording for seven days (**Figure 6A**).

We found that chemogenetic inhibition of PrL during SPS could not rescue the SPS-induced acute changes in sleep/wake duration on day 1 (**Supplementary Figure 5**; 'SPS-D1'-'SD4-D1': mCherry vs. hM4Di). However, inhibition of PrL activity could specifically reverse the SPS-induced acute suppression of NREMS delta power (**Figure 6C-E and Supplementary Figure 6A**; ('SPS-D1'-'SD4-D1')/'SD4-D1': mCherry vs. hM4Di), particularly in the first hour (ZT4) after SPS (**Figure 6C-D**). By contrast, there were no statistically significant differences in other EEG power densities during NREMS, REMS or wake states between mCherry and hM4Di mice (**Supplementary Figure 6**). These results are consistent with the idea that hyper-activities of PrL neurons could result in specific suppression of the NREMS delta power, the best known measurable index of sleep need, immediately after traumatic stress.

To test whether the hyper-activities of PrL neurons during SPS might also result in the

traumatic stress-induced long-term sleep/wake EEG disturbances, we analyzed EEG/EMG data of the mCherry and hM4Di mice on the seventh day after SD4/SPS treatment (**Figure 6A**). Remarkably, we found that chemogenetic inhibition of PrL neurons could abrogate the majority of SPS-induced long-term sleep/wake EEG abnormalities on day 7 (**Figure 6F, 7A and Supplementary Figure 7**; (‘SPS-D7’-‘SD4-D7’)/‘SD4-D7’: mCherry vs. hM4Di). Taken together, these results suggest that SPS-induced hyper-activation of mPFC neurons, particularly in the PrL region, may play a critical role in the development of both short- and long-term sleep-wake EEG disturbances (**Figure 7B**).

Discussion

Traumatic Stress Induces Sleep-wake Architecture and EEG Alterations

The sleep-wake disturbances may be one of the most debilitating symptoms associated with PTSD (Pawlyk et al., 2008). In this study, we adopted the well-established SPS paradigm to investigate the effects of traumatic stress on the sleep-wake architecture in the isogenic mouse model. In accordance with what Liberzon and colleagues had originally observed in SPS rats (Yamamoto et al., 2009), we showed that SPS mice also exhibited higher immobility time than control mice in forced swim test (FST), but similar immobility time in tail suspension test (TST) on the seventh day after SD4/SPS treatment (Supplementary Figure 4E). This result, together with our findings that SPS mice exhibited robust short and long-term sleep disturbances–a core symptom of PTSD patients–further validated the cross-species utility of mouse SPS-PTSD model. Because of the isogenic background and many genetics tools available, the mouse SPS-PTSD model offers unique advantages than the rat SPS-PTSD model in future mechanistic studies of traumatic stress-induced sleep disturbances.

The acute effects of SPS on sleep-wake architecture have been reported in two previous studies in rats (Nedelcovych et al., 2015; Vanderheyden et al., 2015). Our results are mostly consistent with earlier findings of Nedelcovych et al., but not those

of Vanderheyden et al.. A common finding of all three studies is the significant increase in REMS in the dark phase on the day after SPS (Figure 2). The importance of REMS rebound after acute stress is highlighted by the sleep assessment of humans who experience a traumatic event: those who exhibit long episodes of REMS do not develop PTSD, whereas those who have very short episodes of REMS are likely to develop PTSD (Mellman et al., 2002). Taken together, these findings suggest that REMS rebound during the first dark phase, especially long REMS episodes, may represent an essential adaptive strategy for animals or humans to cope with traumatic stress and avoid the development of PTSD (Stickgold, 2007).

We also observed a specific increase in absolute alpha and beta power of EEG signals during REMS and a broad reduction in absolute EEG power densities during NREMS and wake states after SPS (Figure 3). These significant changes in sleep/wake EEG power spectra may be attributed to traumatic stress-induced dys-regulation of multiple neuronal networks mediated by distinct neuromodulators (Vakalopoulos, 2014). It has been shown that traumatic stress causes serotonin release and regional utilization changes in multiple brain regions (Germain et al., 2008; Pawlyk et al., 2008; Nedelcovych et al., 2015). Several studies have also reported that acute stress increases acetylcholine release in the hippocampus and frontal cortex (Mark et al., 1996) and impairs signaling in the prefrontal cortex (Picciotto et al., 2012). These brain region-specific changes of neuromodulator signaling may lead to acute changes in sleep/wake duration and/or short- and long-term state-dependent EEG abnormalities.

Absolute vs. Relative EEG Power Analysis

Consistent with our previous studies (Funato et al., 2016; Wang et al., 2018), we found that absolute EEG power analysis could consistently outperform relative EEG power analysis by revealing more critical changes in the EEG power spectrum. Moreover, relative EEG power analysis could sometimes distort the data and reach the wrong conclusion (Figure 4E). Thus, we recommend that both absolute and relative EEG power analysis should be performed to obtain comprehensive phenotypic

analysis in future patho/physiological sleep studies, especially when using a longitude experimental design in the isogenic mouse models.

A Mechanistic Link between mPFC Over-activation and Sleep/Wake EEG Disturbances

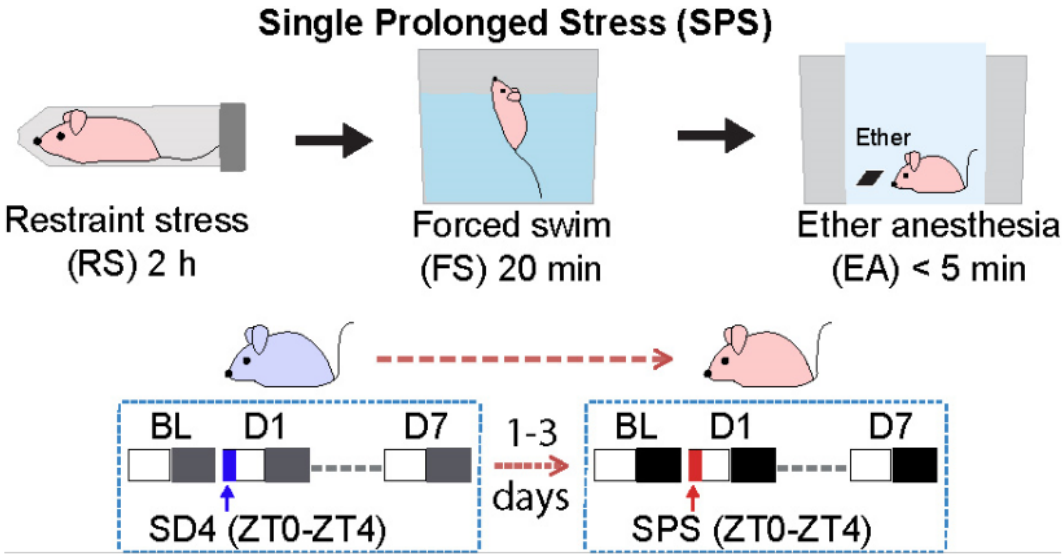
Both acute and chronic stress can cause structural and functional alterations of the mPFC, resulting in dys-regulation of the cognitive-emotional control and threat extinction (Holmes and Wellman, 2009; Herringa, 2017; Heyn et al., 2019). Although sleep deprivation itself changes the neuronal properties of the mPFC (Winters et al., 2011; Ren et al., 2018), the persistent mPFC activity after SPS may reflect reactivation of the memory trace during slow wave sleep (SWS)/NREMS (Peyrache et al., 2009). Mounting evidence suggest that neural patterns reflecting previously acquired information are replayed to facilitate information exchange between the hippocampus and neocortex, supporting consolidation of short-term memories into long-term memories during SWS (Peyrache et al., 2009).

Our chemogenetic inhibition experiments strongly suggest that the hyper-activation of mPFC neurons during SPS may mediate specific suppression of NREMS delta power immediately after SPS treatment (Figure 6), and eventually lead to the long-term sleep/wake EEG abnormalities (Figure 7). To our best knowledge, our study represents the first attempt to establish such a causality link between dysfunction of a specific brain region and traumatic stress-induced sleep/wake EEG abnormalities. Recent studies suggest that the mPFC contains a heterogeneous neural population, including the pyramidal neurons and interneurons that may exert opposite regulation on EEG activities. Whereas pyramidal neuronal activity results in cortical activation and desynchronization, inhibitory interneurons that express somatostatin (SOM) are involved in the generation and propagation of slow waves characteristic of NREM sleep (Funk et al., 2017). Although the detailed mechanism by which the mPFC responds to SPS is unclear, we found that more than 95% of c-fos-expressing neurons in the mPFC are excitatory neurons (Figure 5E, F), suggesting that hyper-activities of pyramidal neurons, rather than interneurons such as SOM+ or parvalbumin positive

interneurons, in the mPFC are probably involved in the acute suppression of NREMS delta power. However, future studies are needed to investigate the precise roles of different types of mPFC neurons in the SPS-induced sleep-wake EEG disturbances as the chemogenetic inhibition approach in our study result in the inhibition of all neuronal populations.

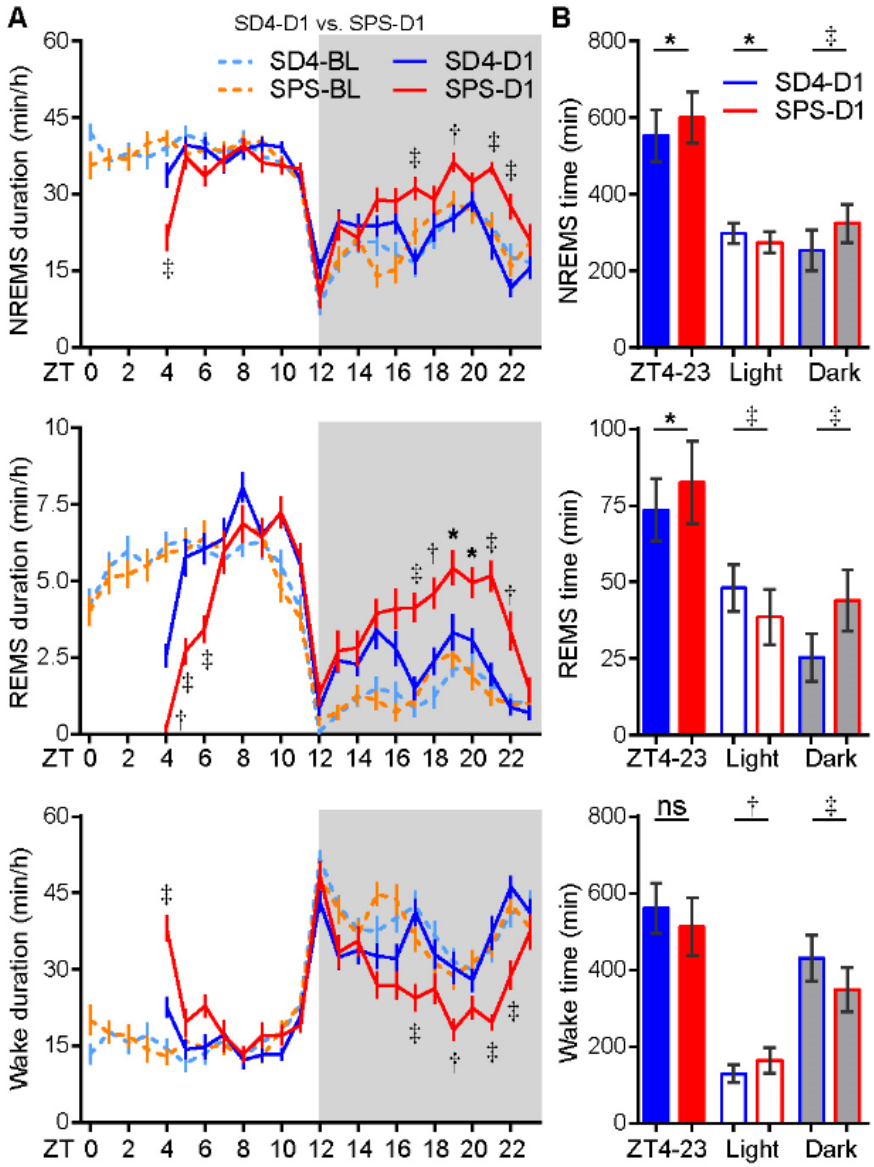
Both reduced and increased delta power activity during NREMS have been reported in PTSD patients (Woodward et al., 2000; Germain et al., 2006; Insana et al., 2012; de Boer et al., 2020; Wang et al., 2020). Thus, the SPS mouse model may recapitulate the symptoms of the subset of PTSD patients showing reduced NREMS delta power (Woodward et al., 2000; de Boer et al., 2020; Wang et al., 2020). In our study, we found that chemogenetic inhibition of the mFPC activity could specifically reverse the SPS-induced acute suppression of delta power during NREMS and most of the long-term sleep/wake EEG abnormalities. Moreover, sleep deprivation immediately after trauma, which normally elevates NREMS delta power during recovery sleep, has been reported as an effective intervention for attenuating PTSD-like behavioral disruptions (Cohen et al., 2012; Cohen et al., 2017). These observations underscore the importance of sleep-dependent processes of neural reactivation in the development of PTSD (Cohen et al., 2012; Cohen et al., 2017). Our findings may suggest the mPFC as an attractive target for the development of effective therapeutics for traumatic stress-induced psychiatric disorders, such as PTSD.

FIGURE 1| Experimental design for studying single prolonged stress (SPS)-induced sleep-wake disturbances.



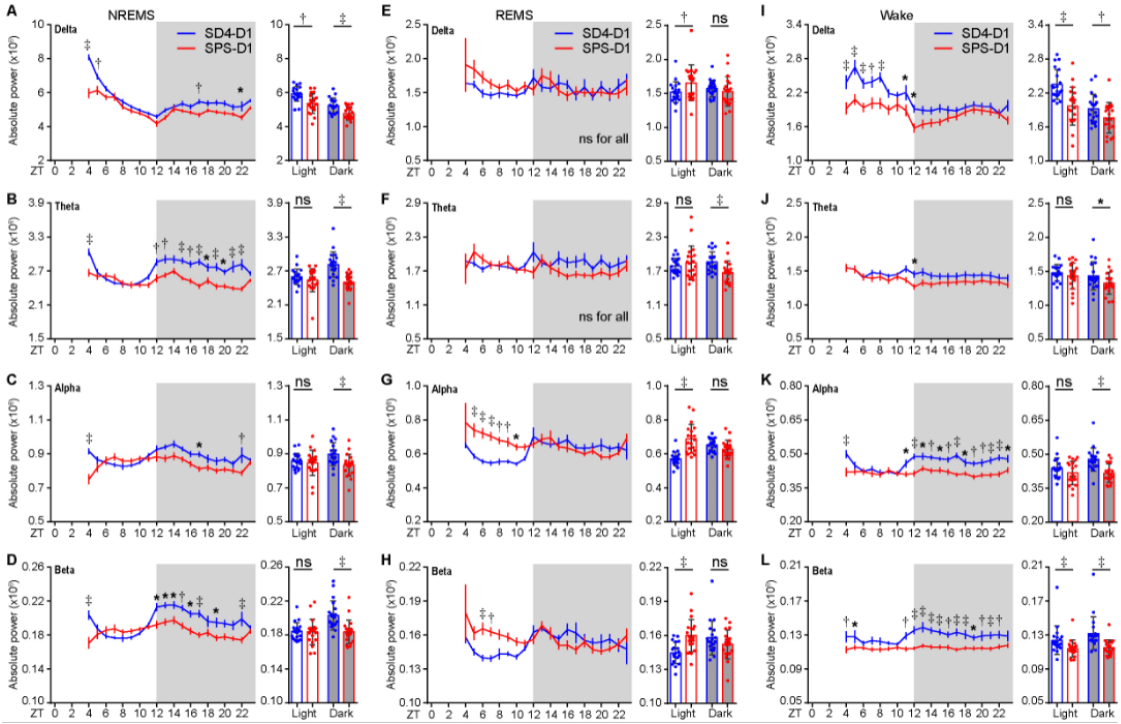
The same group of C57BL/6N male mice (n = 20) were subjected to seven day EEG/EMG recording after sleep deprivation (SD4, ZT0-ZT4), and followed by seven day EEG/EMG recording after single prolonged stress (SPS, ZT0-ZT4).

FIGURE 2| Traumatic stress induces acute changes in sleep/wake duration.



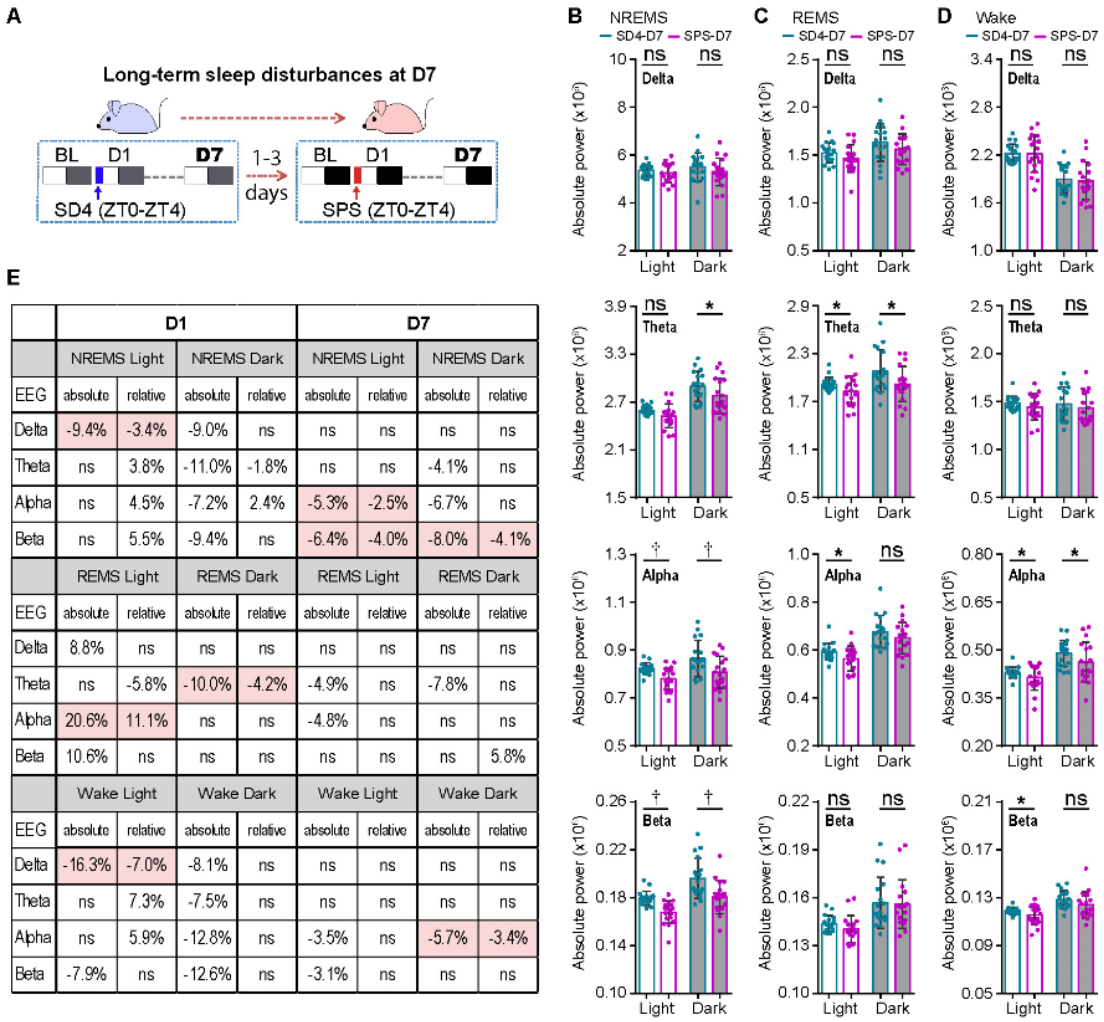
(A) Analysis of NREMS, REMS, or wake duration in every hour on the day before (SD4-BL and SPS-BL) or after (SD4-D1 and SPS-D1) SD4/SPS treatment. (B) Quantitative analysis of total NREMS, REMS, or wake time on the day after SD4 or SPS treatment (SD4-D1 vs. SPS-D1). Mean ±s.e.m., two-way ANOVA, Sidak's test (A); Mean ± s.d., paired *t*-test, two-tailed (B). *, P < 0.05;†, P < 0.01; ‡, P < 0.001; ns, P > 0.05.

FIGURE 3 | Traumatic stress induces acute changes in sleep/wake EEG power spectrum.



(A-L) Analysis of mean absolute EEG power density in every hour (left, hourly) or in the light/dark phase (right) during NREMS (A-D), REMS (E-H) and wake (I-L) states of test mice (n = 20) on the day after SD4/SPS treatment (SD4-D1 vs. SPS-D1). Mean ± s.e.m., two-way ANOVA, Sidak's test (for hourly analysis); Mean ± s.d., paired *t*-test, two-tailed (for mean analysis). *, P <0.05; †, P < 0.01; ‡, P < 0.001; ns, P > 0.05.

FIGURE 4 | Traumatic stress induces long-term alterations of sleep/wake EEG power spectrum.

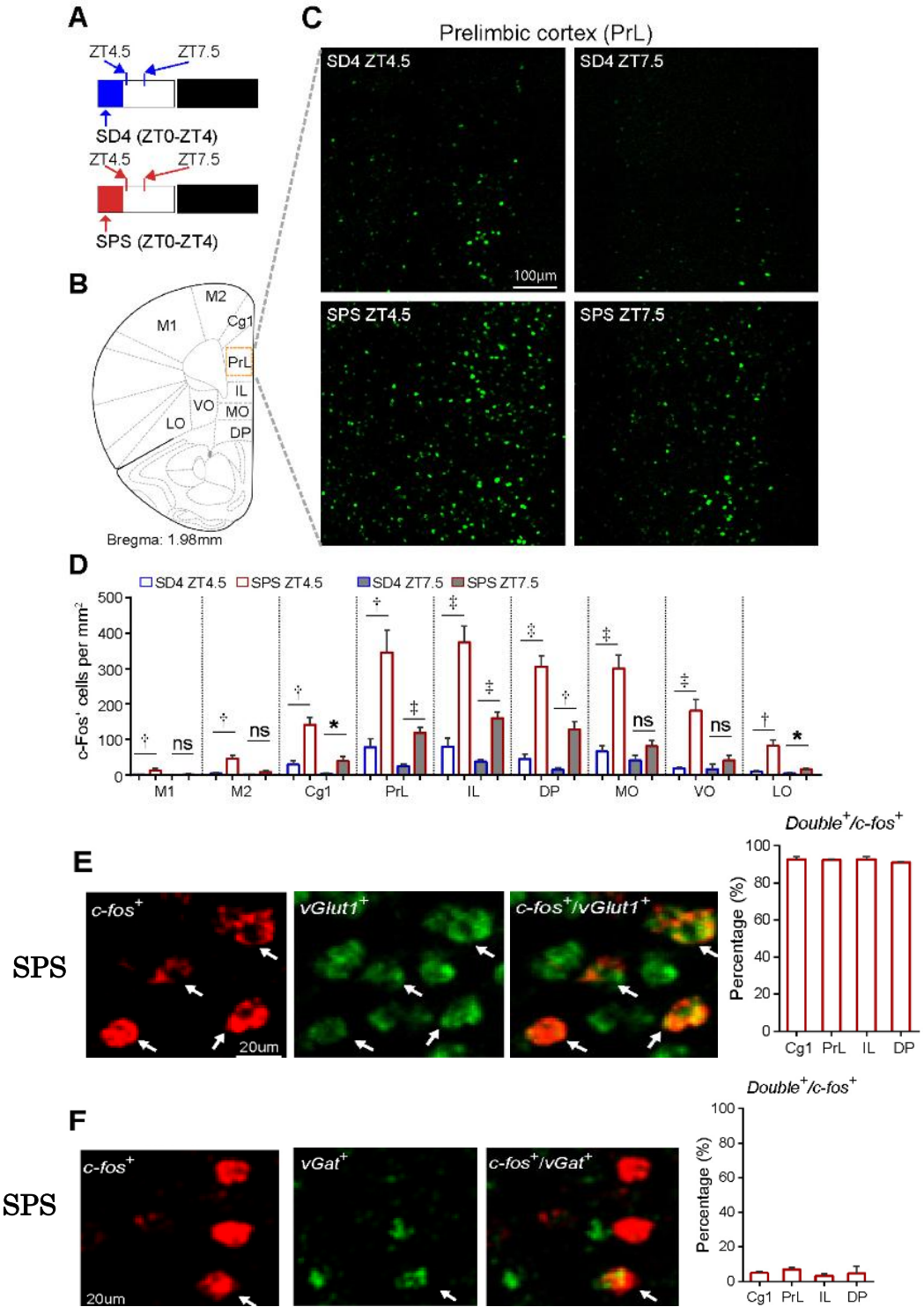


E

	D1				D7			
	NREMS Light		NREMS Dark		NREMS Light		NREMS Dark	
EEG	absolute	relative	absolute	relative	absolute	relative	absolute	relative
Delta	-9.4%	-3.4%	-9.0%	ns	ns	ns	ns	ns
Theta	ns	3.8%	-11.0%	-1.8%	ns	ns	-4.1%	ns
Alpha	ns	4.5%	-7.2%	2.4%	-5.3%	-2.5%	-6.7%	ns
Beta	ns	5.5%	-9.4%	ns	-6.4%	-4.0%	-8.0%	-4.1%
	REMS Light		REMS Dark		REMS Light		REMS Dark	
EEG	absolute	relative	absolute	relative	absolute	relative	absolute	relative
Delta	8.8%	ns	ns	ns	ns	ns	ns	ns
Theta	ns	-5.8%	-10.0%	-4.2%	-4.9%	ns	-7.8%	ns
Alpha	20.6%	11.1%	ns	ns	-4.8%	ns	ns	ns
Beta	10.6%	ns	ns	ns	ns	ns	ns	5.8%
	Wake Light		Wake Dark		Wake Light		Wake Dark	
EEG	absolute	relative	absolute	relative	absolute	relative	absolute	relative
Delta	-16.3%	-7.0%	-8.1%	ns	ns	ns	ns	ns
Theta	ns	7.3%	-7.5%	ns	ns	ns	ns	ns
Alpha	ns	5.9%	-12.8%	ns	-3.5%	ns	-5.7%	-3.4%
Beta	-7.9%	ns	-12.6%	ns	-3.1%	ns	ns	ns

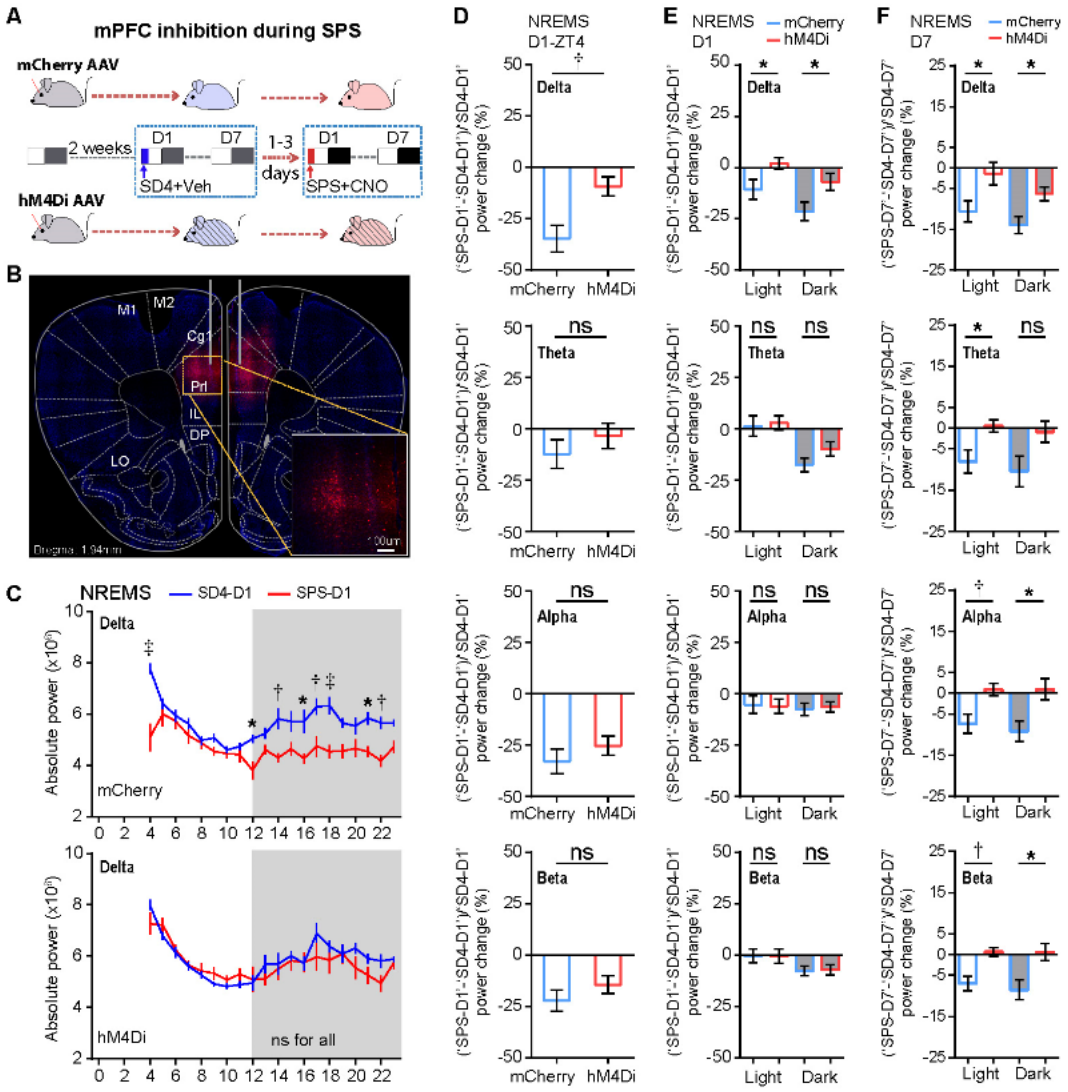
(A) A schematic of sleep-wake analysis at D7 after SD4/SPS treatment. (B-D) Analysis of mean absolute EEG power density in NREMS (B), REMS (C) and wake (D) states of the same test mice on day 7 after SD4/SPS treatment (SD4-D7 vs. SPS-D7). (E) A table comparing the specific change ratios of the delta, theta, alpha, and beta power bands of EEG signals detected by absolute and relative EEG power analysis on D1 [('SPS-D1'-'SD4-D1')/'SD4-D1'] and on D7 [('SPS-D7'-'SD4-D7')/'SD4-D7'] after SD4/SPS treatment. Mean ± s.d., paired *t*-test, two-tailed (B-D). *, P < 0.05; †, P < 0.01; ns, P > 0.05.

FIGURE 5 | Traumatic stress induces persistent c-Fos expression in the mPFC neurons.



Experimental design for mapping brain regions showing abnormal c-Fos expression after SPS relative to SD4 treatment. Mouse brains were harvested at 30 min (ZT4.5) or 3.5 h (ZT7.5) after SPS or SD4 treatment. (B) A schematic map of mouse prefrontal cortex showing different sub regions. (C) Representative images showing c-Fos immunohistochemistry in the prelimbic cortex (PrL) of SPS/SD4 mice at ZT4.5 and ZT7.5. (D) Quantitative analysis of c-Fos–expressing neurons at ZT4.5 and ZT7.5 after SPS/SD4 treatment in different subregions of prefrontal cortex (n=5). Mean ± s.e.m., unpaired *t*-test, two-tailed. (E-F) Fluorescent two-color in situ hybridization staining and quantitation of *vGlut1*+ (E) or *vGat*+ (F), and *c-fos* + 10 double positive neurons after SPS treatment. *vGlut1*, n=3; *vGat*, n=4. Mean ± s.e.m.. Primary (M1) and secondary (M2) cortex of motor cortex; cingulate (Cg1), prelimbic (PrL), infralimbic (IL) and dorsal peduncular (DP) cortex within mPFC; medial (MO), ventral (VO) and lateral (LO) part of the orbitofrontal cortex (OFC). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

FIGURE 6 | Chemogenetic inhibition of mPFC-PrL neurons specifically reverses SPS-induced acute suppression of NREMS delta power and long-term EEG alterations.



(A) Experimental design for chemogenetic inhibition of mPFC-PrL during SPS treatment. Two groups of C57BL/6N male mice (n =9) were bilaterally injected into the PrL region of mPFC with AAV2/9-mCherry (mCherry) or AAV2/9-hM4Di-mCherry (hM4Di), respectively. All mice were subjected to seven day EEG/EMG recording after sleep deprivation (SD4, ZT0-ZT4), and subsequently subjected to seven day EEG/EMG recording after SPS (ZT0-ZT4). Intraperitoneal injection of vehicle (0.9% saline) during SD4 or CNO (3 mg/kg) during SPS was administered at ZT0 and ZT3.5. (B) Representative image showing correct AAV injection sites marked by mCherry positive cells. (C) Hourly analysis of mean absolute delta power density of NREMS in mCherry (n = 9) and hM4Di (n = 9) mice on the day after SD4/SPS treatment (SD4-D1 vs. SPS4D1). (D-E) Comparison of the change ratio [('SPS-D1'-'SD4-D1')/'SD4-D1']% in the mean absolute NREMS EEG power density of mCherry and hM4Di mice at ZT4 (D), and in the light or dark phase (E). (F) Comparison of the change ratio [('SPS-D7'-'SD4-D7')/ 'SD4-D7']% in the mean absolute NREMS EEG power density of mCherry and hM4Di mice in the light or dark phase on day 7 after SD4/SPS treatment. Mean ± s.e.m., two-way ANOVA, Sidak's test (C); Mean ± s.e.m., unpaired *t*-test, two-tailed (D- F). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

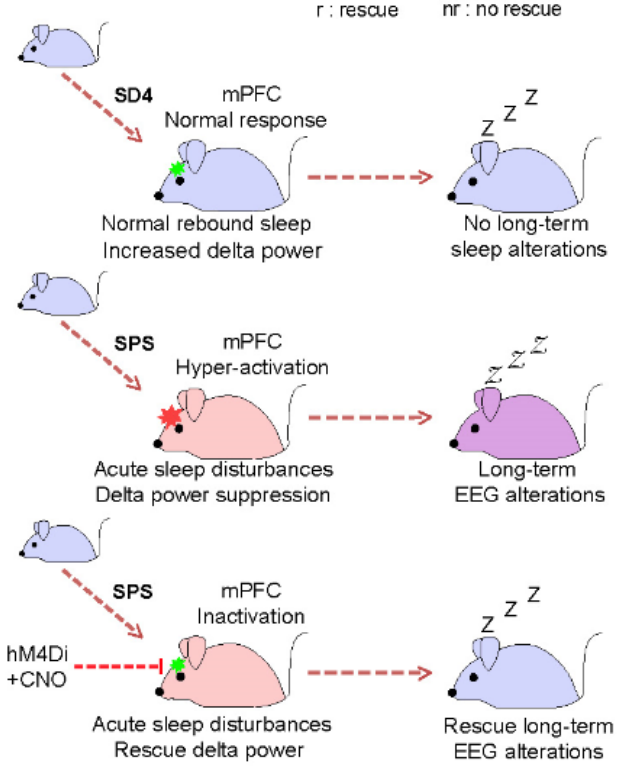
FIGURE 7 | Hyper-activation of mPFC underlies traumatic stress-induced sleep-wake EEG disturbances.

A

		D1									D7					
		NREMS			REMS			Wake			NREMS		REMS		Wake	
EEG Power		ZT4	L	D	ZT4	L	D	ZT4	L	D	L	D	L	D	L	D
Delta	mCherry	↓	–	↓	–	–	–	↓	↓	–	↓	↓	↓	–	↓	–
	hM4Di	r	–	r	–	–	–	nr	nr	–	r	r	nr	–	r	–
Theta	mCherry	↓	–	↓	–	–	–	–	–	–	↓	↓	–	–	–	–
	hM4Di	nr	–	nr	–	–	–	–	–	–	r	nr	–	–	–	–
Alpha	mCherry	↓	–	↓	–	↑	–	↓	–	↓	↓	↓	↓	–	↓	↓
	hM4Di	nr	–	nr	–	nr	–	nr	–	nr	r	r	r	–	r	nr
Beta	mCherry	↓	–	↓	–	↑	–	–	–	↓	↓	↓	–	–	↓	↓
	hM4Di	nr	–	nr	–	nr	–	–	–	nr	r	r	–	–	r	nr

L: light phase D: dark phase ↑ increased ↓ decreased – no change
r : rescue nr : no rescue

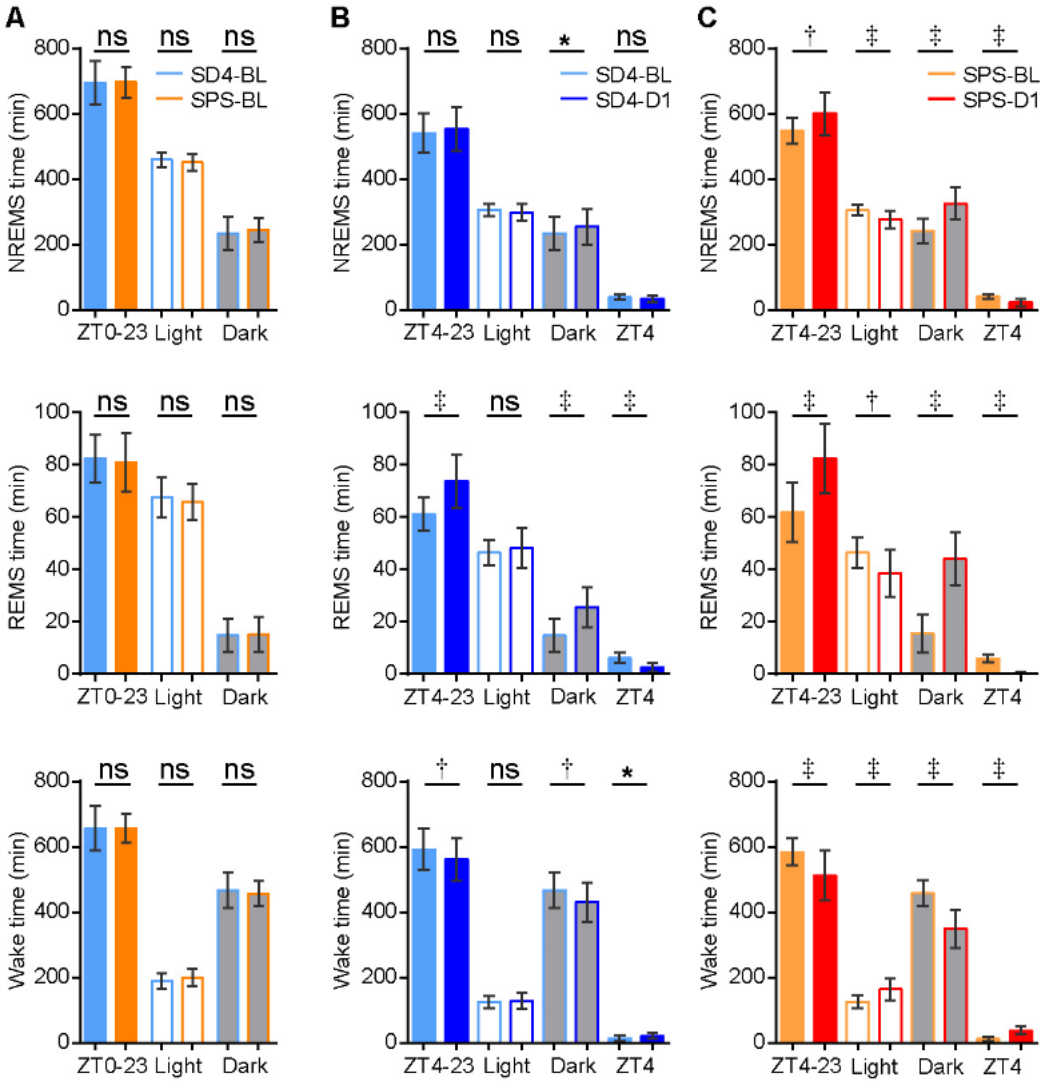
B



(A) A table summarizing the results of chemogenetic inhibition experiments. Highlighted regions indicate the specific SPS-induced short-term and long-term EEG abnormalities that can be rescued by chemogentic inhibition of mPFC. (B) A model showing that hyper-activation of mPFC contributes critically to the SPS-induced sleep-wake EEG disturbances.

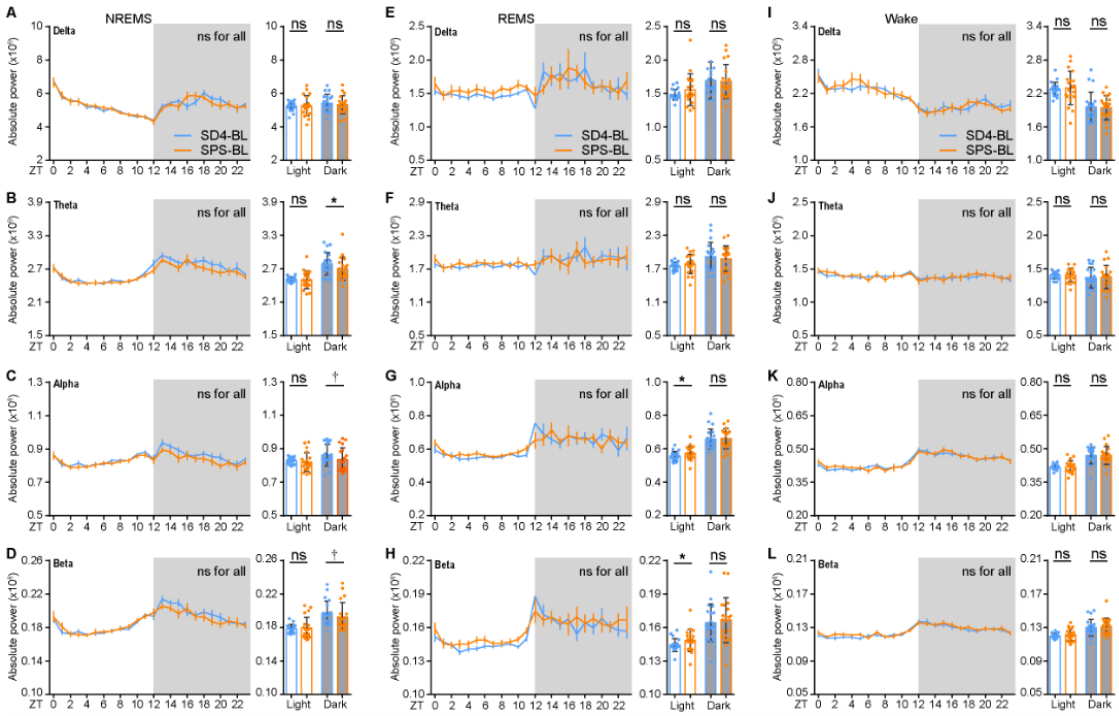
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 |



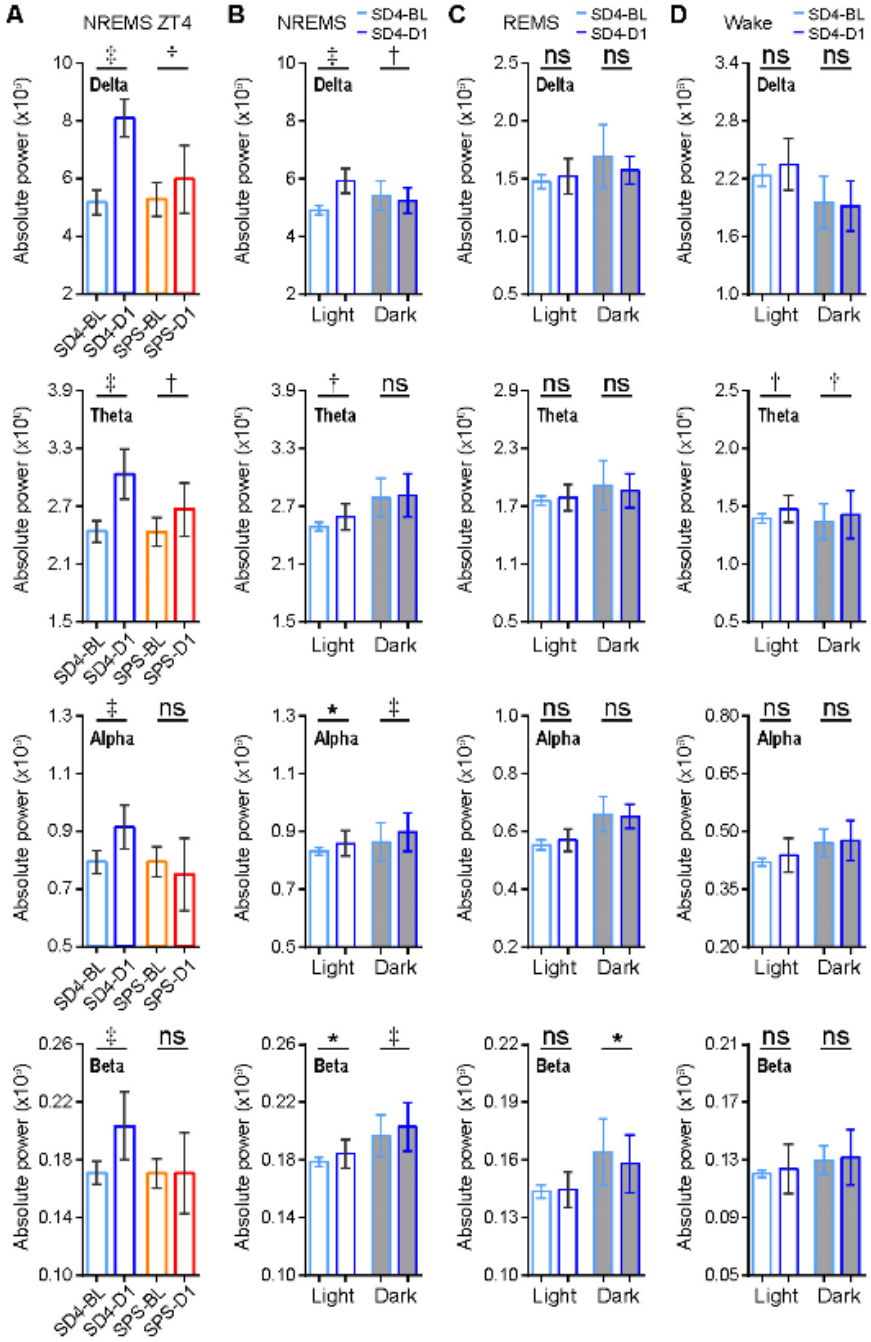
(A) Quantitative analysis of total NREMS, REMS, or wake time on the day before SD4/SPS treatment (SD4-BL vs. SPS-BL). (B-C) Quantitative analysis of NREMS, REMS, or wake time on the day before and after 4h sleep deprivation (SD4-D1 vs. SD4-BL) (B), and on the day before and after SPS treatment (SPS-D1 vs. SPS-BL) (C). n = 20, Mean ± s.d., paired t-test, two-tailed (A-C). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

Supplementary Figure 2 |



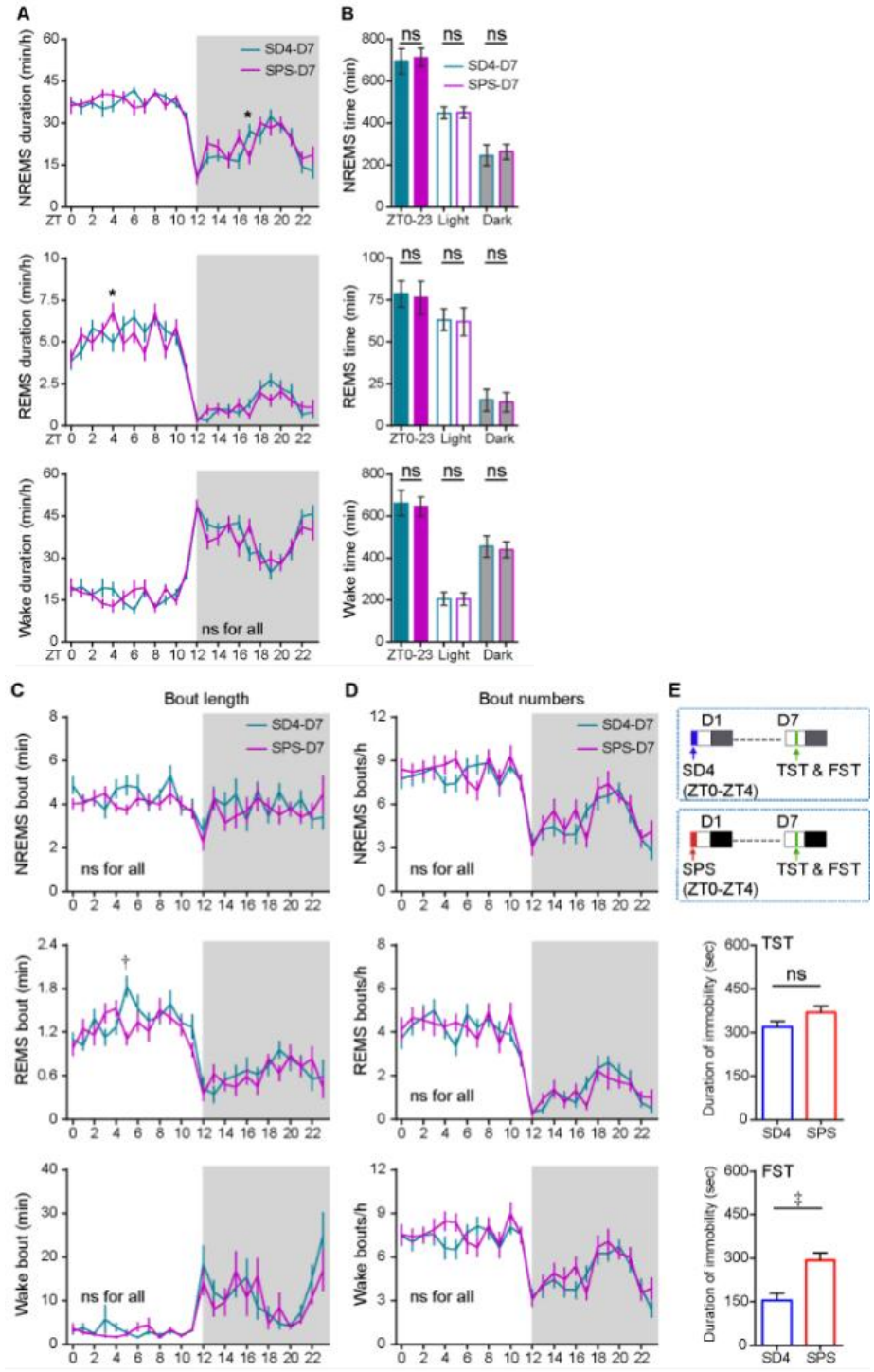
(A-L) Analysis of mean absolute EEG power density in every hour (left, hourly) or in the light/dark phase (right) in NREMS (A-D), REMS (E-H) and wake (I-L) states of test mice (n = 20) on the day before SD4/SPS treatment (SD4-BL vs. SPS-BL). Mean ±s.e.m., two-way ANOVA, Sidak's test (for hourly analysis); Mean ± s.d., paired t-test, two-tailed (for mean analysis). *, $P < 0.05$; †, $P < 0.01$; ns, $P > 0.05$.

Supplementary Figure 3 |



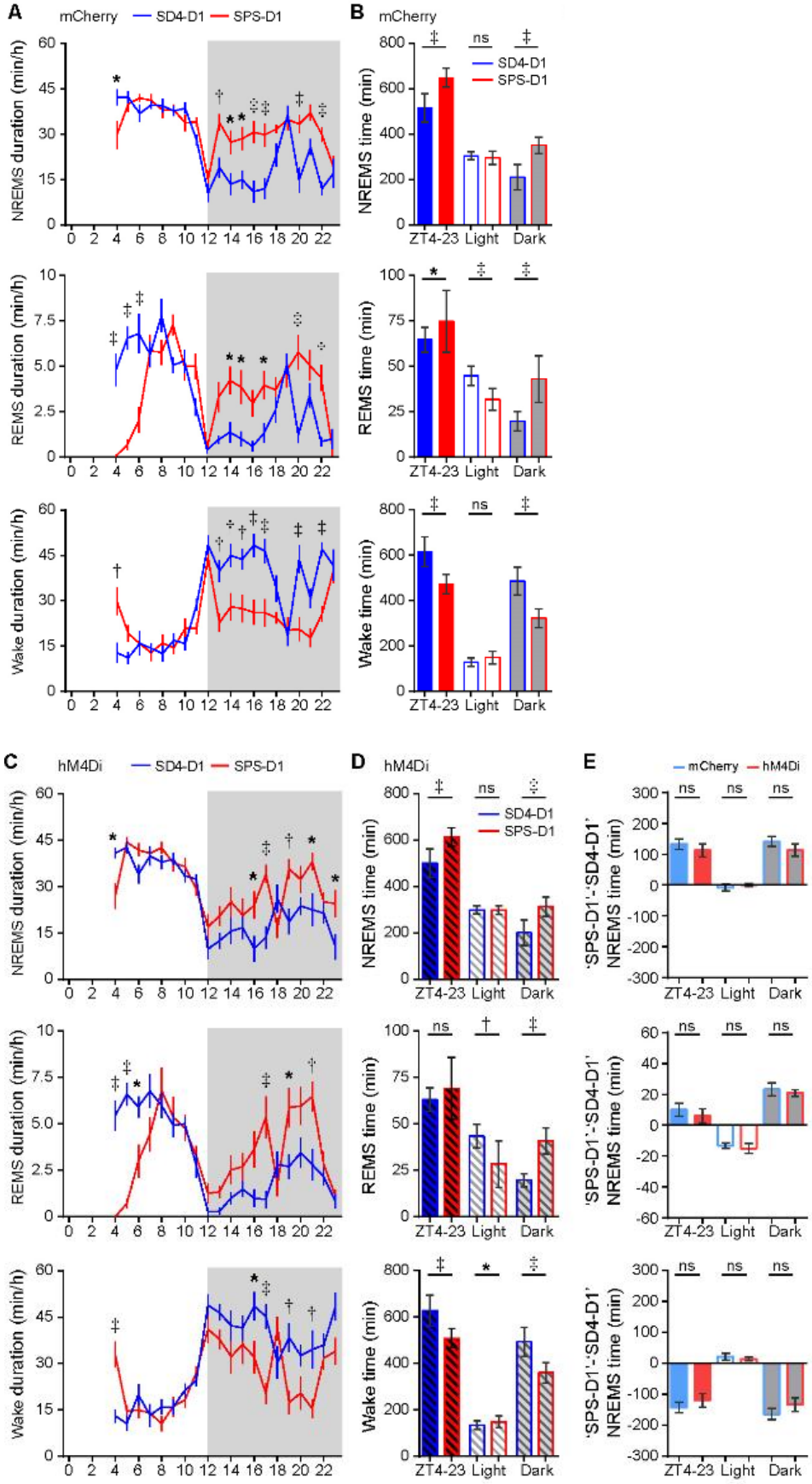
(A) Analysis of mean absolute NREMS ZT4 EEG power density on the day before and after SD4/SPS treatment (SD4-D1 vs. SD4-BL; SPS-D1 vs. SPS-BL), (n=20). (B-D) Analysis of mean absolute EEG power density in the light/dark phase during NREMS (B), REMS (C) and wake (D) states of test mice (n = 20) on the day before and after SD4 treatment (SD4-D1 vs. SD4-BL). Mean ± s.e.m., unpaired t-test, two-tailed (A); Mean ± s.d., paired t-test, two-tailed (B-D). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

Supplementary Figure 4 |



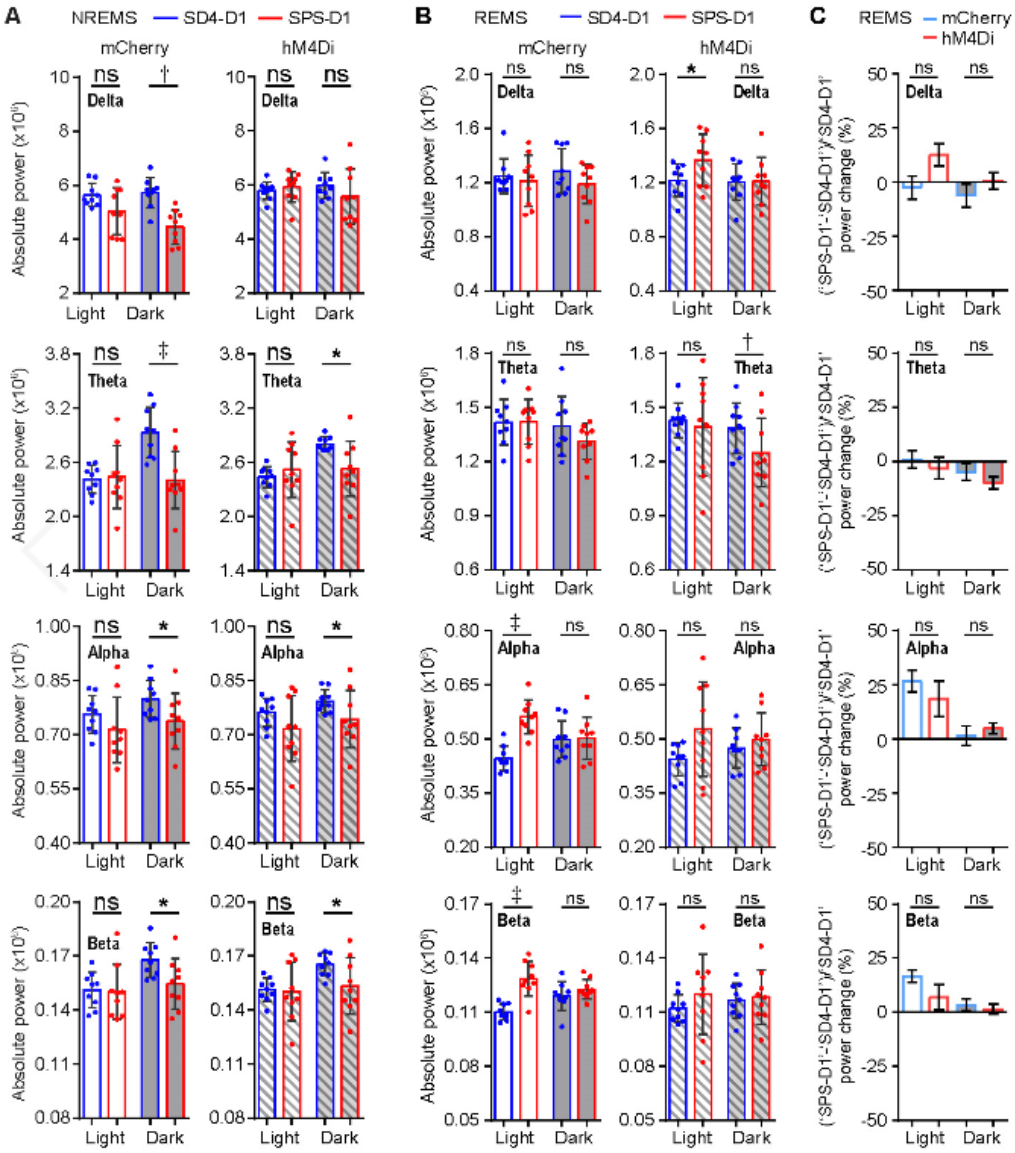
(A–B) Hourly (A) and quantitative (B) analysis of NREMS, REMS and wake duration of test mice (n = 19) on day 7 after SD4 or SPS treatment (SD4-D7 vs. SPS-D7). (C–D) Hourly analysis of episode duration (C) or episode number (D) of NREMS, REMS and wake states of test mice (n = 19) on D7 after SPS treatment (SD4-D7 vs. SPS-D7). (E) Comparison of immobility time in the tail suspension test (TST) and forced swimming test (FST) on day 7 after SPS/SD4 treatment. SD4, n = 17; SPS, n= 12. Mean ± s.e.m., two-way RM ANOVA, Sidak's test (A, C, D); Mean ± s.d., paired t-test, two-tailed (B); Mean ± s.e.m., unpaired t-test, two-tailed (E). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

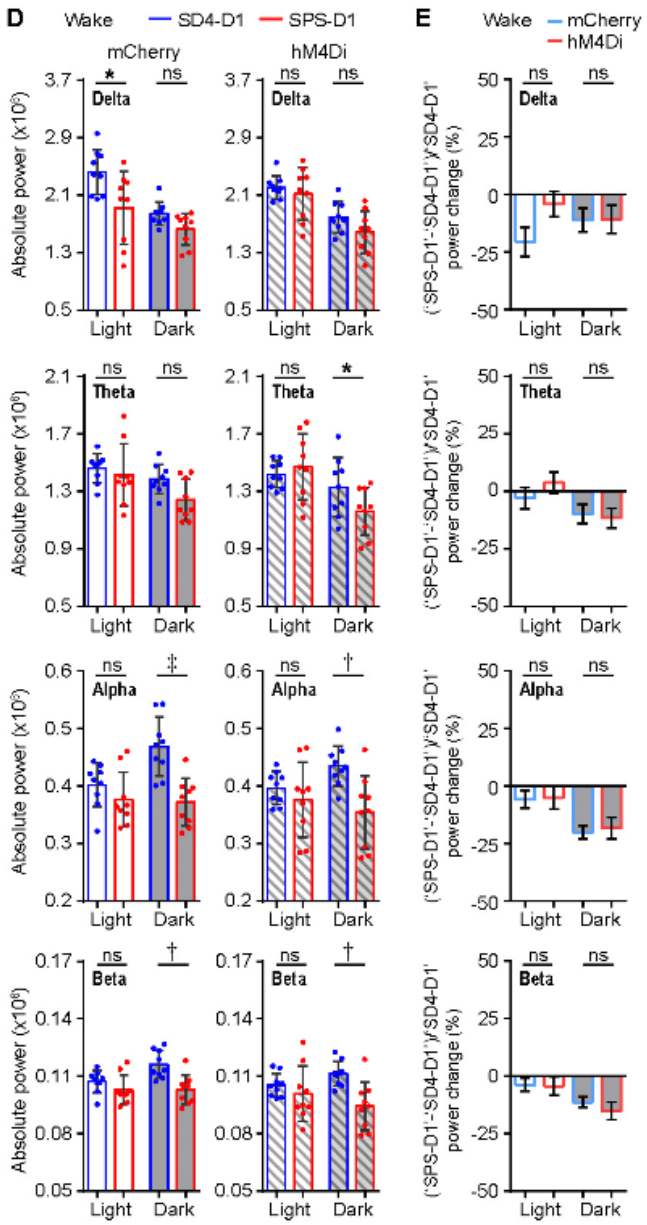
Supplementary Figure 5 |



(A-B) Hourly (A) and quantitative (B) analysis of NREMS, REMS and wake duration of mCherry (n = 9) mice on the day after SD4/SPS treatment (SD4-D1 vs. SPS- D1). (C-D) Hourly (C) and quantitative (D) analysis of NREMS, REMS and wake duration of hM4Di (n = 9) mice on the day after SD4/SPS treatment (SD4-D1 vs. SPS-D1). (E) Comparison of the difference ('SPS-D1'-'SD4-D1') in NREMS, REMS or wake time of mCherry (n = 9) and hM4Di mice (n = 9) on the day after SD4/SPS treatment. Mean ± s.e.m., two-way RM ANOVA, Sidak's test (A, C); Mean ± s.d., paired t-test, two-tailed (B, D); Mean ± s.e.m., unpaired t-test, two-tailed (E). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

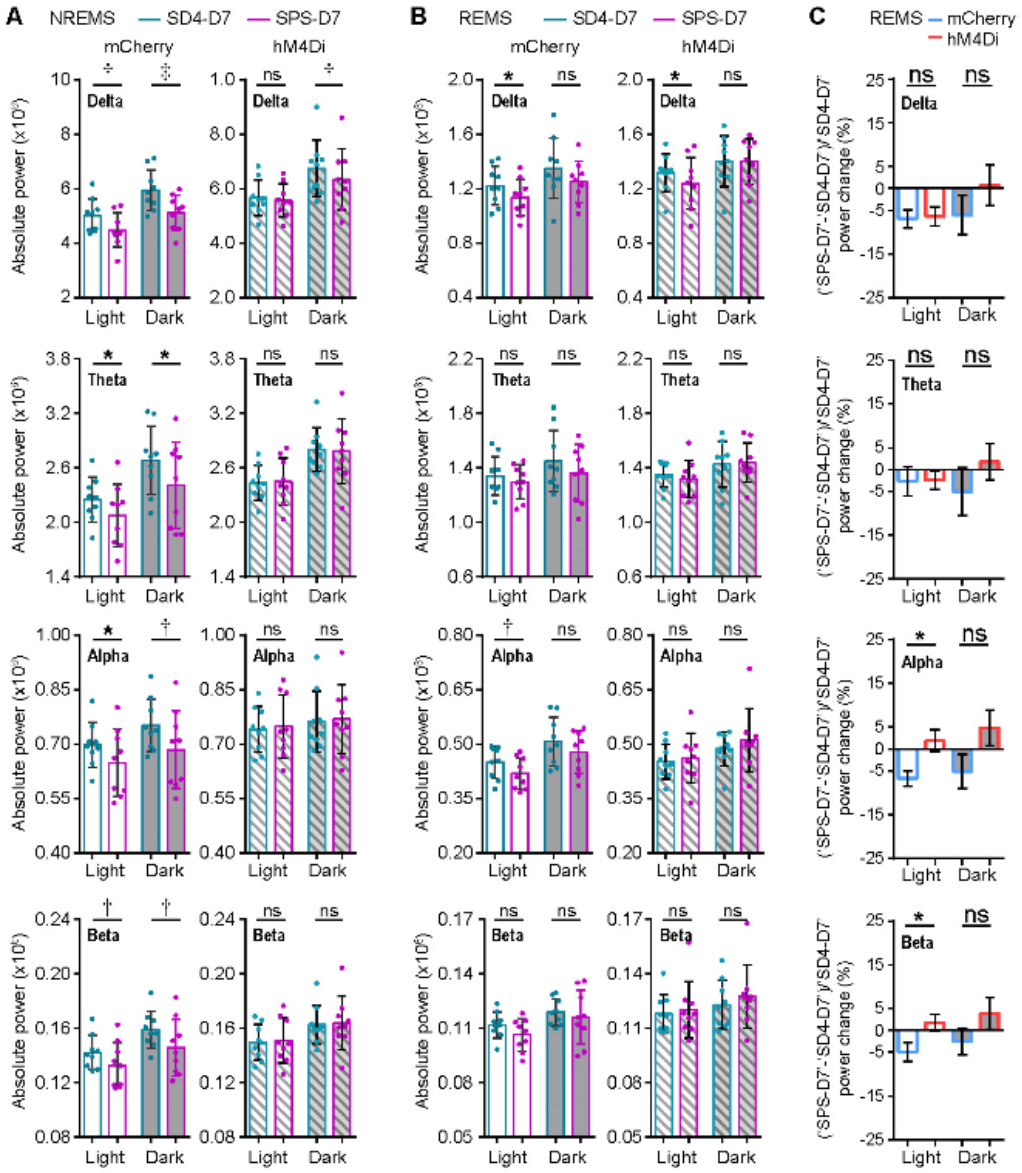
Supplementary Figure 6 |

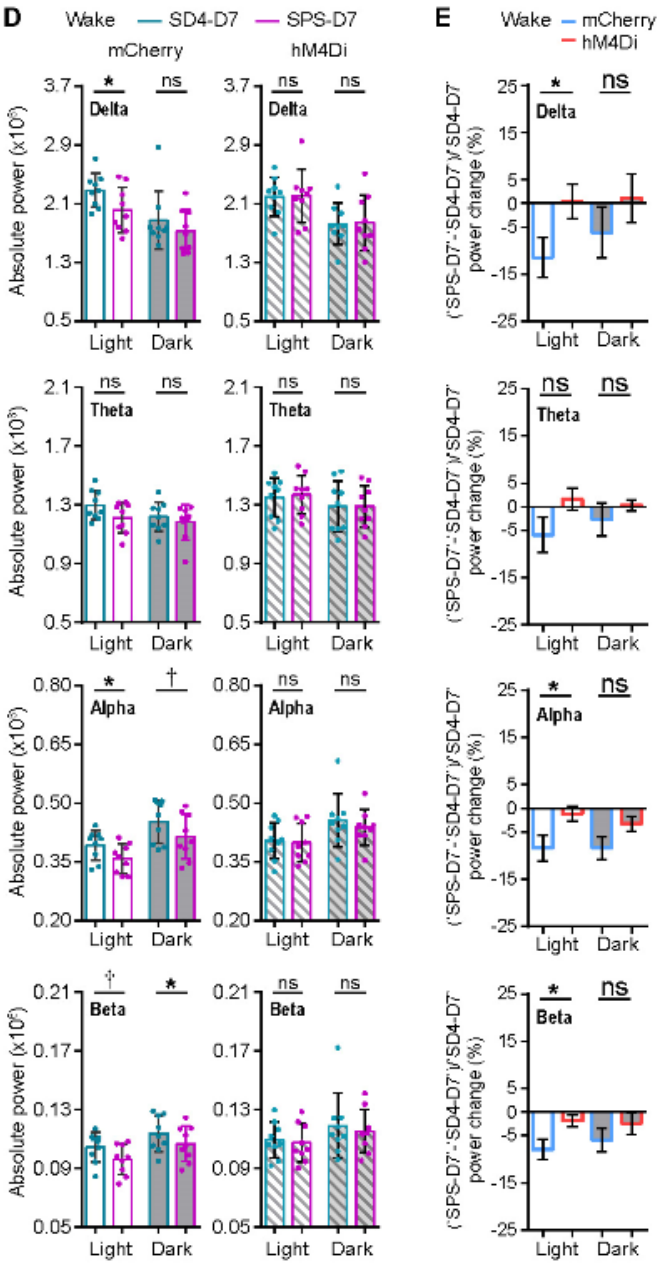




(A-E) Analysis of mean absolute NREMS (A), REMS (B) or wake (D) EEG power density of mCherry (n = 9) and hM4Di (n = 9) mice on the day after SD4/SPS treatment (SD4-D1 vs. SPS-D1). Comparison of the change ratio [('SPS-D1'-'SD4-D1')/'SD4- D1']% in the mean absolute REMS (C) and wake (E) EEG power density of mCherry and hM4Di mice (n=9) in the light or dark phase. Mean ± s.d., paired t-test, two-tailed (A, B, D); Mean ± s.e.m., unpaired t-test, two-tailed (C, E). *, P < 0.05; †, P < 0.01; ‡, P < 0.001; ns, P > 1 0.05.

Supplementary Figure 7 |





(A-E) Analysis of mean absolute EEG power density during NREMS (A), REMS (B) and wake (D) states of mCherry (n = 9) and hM4Di (n = 9) mice on day 7 after SD4/SPS treatment (SD4-D7 vs. SPS-D7). Comparison of the change ratio [('SPS-D7'-'SD4-D7')/ 'SD4-D7']% in the mean absolute EEG power density during REMS (C) and wake (E) states of mCherry (n = 9) and hM4Di (n = 9) mice in the light or dark phase on day 7 after SD4/SPS treatment. Mean ± s.d., paired t-test, two-tailed (A, B, D); Mean ± s.e.m., unpaired t-test, two-tailed (C, E). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; ns, $P > 0.05$.

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Source

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