INSIGHTS INTO PARADOXICAL (REM) SLEEP HOMEOSTATIC REGULATION IN MICE USING AN INNOVATIVE AUTOMATED SLEEP DEPRIVATION METHOD.

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ABSTRACT

Identifying the precise neuronal networks activated during paradoxical sleep (PS, also called REM sleep) has been a challenge since its discovery. Similarly, our understanding of the homeostatic mechanisms regulating PS, whether through external modulation by circadian and ultradian drives, or via intrinsic homeostatic regulation is still limited, largely due to interfering factors rendering the investigation difficult. Indeed, none of the studies published so far were able to manipulate PS without significantly altering slow-wave-sleep and/or stress level, thus introducing a potential bias in the analyses.

With the aim of achieving a better understanding of PS homeostasis, we developed a new method based on automated scoring of vigilance states - using EEG and EMG features -, and which involves closed-loop PS deprivation through the induction of cage floor movements when PS is detected. Vigilance states were analyzed during 6h and 48h of PS deprivation as well as their following recovery periods.

Using this new automated methodology, we were able to deprive mice of PS with high efficiency and specificity, for short or longer periods of time, observing no sign of stress (as evaluated by plasma corticosterone level and sleep latency) and requiring no human intervention or environmental changes. We show here that PS can be homeostatically modulated and regulated while no significant changes is induced on slow-wave-sleep and wakefulness, with a PS rebound duration depending on the amount of prior PS deficit. We also show that PS-interval duration is not correlated with prior PS episode duration in the context of recovery from PS deprivation.

Keywords

Sleep; Stress; Oscillation; EEG ; polysomnography; homeostasis; REM sleep; deprivation method

Statement of significance:

A large body of controversial literature has been produced trying to reveal the nature of paradoxical sleep (REM sleep) homeostatic regulation. In these studies, paradoxical sleep was challenged by different means, using total sleep deprivation, more or less specific PS deprivation and most of the time inducing a fair amount of stress. Here we report using an innovative method allowing to highly specifically deprives mice of paradoxical sleep without significantly modifying other vigilance states, that paradoxical sleep homeostatic regulation depends only on prior amount of paradoxical sleep deficit. Gaining a better understating on how paradoxical sleep is regulated is crucial in helping to treat its co-morbid dysregulation in diseases such as anxiety disorders.

INTRODUCTION

Paradoxical sleep, also called REM sleep for rapid-eye-movement sleep, is a vigilance state which, in rodents, is characterized by a low voltage, fast rhythms EEG activity highly enriched in the theta frequency, coupled with a loss of awareness and consciousness of the environment, rapid eye movements (although eyes are closed), complete muscle atonia occasionally accompanied by brief distal twitches, and irregular heart and respiratory rates [1].

Identifying the precise neuronal networks activated during paradoxical sleep (also called REM sleep) has been a challenge since its discovery. Similarly, our understanding of the homeostatic mechanisms regulating paradoxical sleep, whether through external modulation by circadian and ultradian drives, or via intrinsic homeostatic regulation (ie: the need to compensate after a loss, striving to maintain sleep need within an acceptable range) is still limited.

In a recent elegant study, Weber et al [2] proposed that GABAergic neurons of the ventrolateral periaqueductal gray could control the ultradian occurrence of PS. Convergent data show that the circadian control of PS by the masterclock might depend on the action of the orexin/hypocretin neuropeptides [3-5]. However, our understanding of the homeostatic mechanisms regulating PS is still obscure, largely due to interfering factors rendering the investigation difficult. Indeed, a large body of controversial literature has been produced based on total sleep deprivation or PS deprivation of different durations and using different methodologies (for review, [6]). It has been argued that PS homeostatic regulation is dependent on previous NREM sleep (non-REM sleep also called SWS for slow-wave-sleep) [7] or on both wakefulness and SWS [8]. However, none of the studies published so far were able to manipulate PS without a significant alteration of SWS sleep (its quality or quantity) and/or stress level (as evaluated by measuring plasma corticosterone concentration) thus introducing potential biases in the analyses. In fact, in all of these studies, PS homeostasis was challenged by deprivation methodologies more or less specific to PS, the most common ones being the “single or multiple-platforms over water”, the disk-over-water methods and the “gentle handling” (see review by [6]). The platforms and disk-over-water methods are stressful [9, 10], are often used for long periods of time (48 to 96h) [11, 12] and are associated with an unwelcoming environment [13, 14]. If gentle handling seems to be less stressful, it is highly time-consuming, thus often used for short durations; it is based on an online human subjective interpretation of PS boundaries, relies on the experimenter’s capacity for attention over time, and requires human intervention [15]. All these parameters may also introduce biases.

Thus, to properly evaluate PS homeostasis, we developed a new method allowing automated PS deprivation [16] with high efficiency and specificity, for short or longer periods of time, mice showing no sign of stress (as evaluated by level of plasma corticosterone, fur quality and latency to fall asleep), and needing no human intervention. Using this new automated methodology in mice, we demonstrate that PS is homeostatically modulated and regulated, with the PS rebound reflecting essentially the amount of PS lost.

METHODS

Animals

Adult male C57Bl/6J mice (n = 70; 25-30g; 11-13 weeks of age; Charles River, France) were used. The experimental procedures were approved by the Ethics Research Committee of University-Lyon1 in accordance with the European guidelines for care in animal research (protocol # BH200609). Mice were housed in groups of siblings before surgery and in individual barrels after. Mice were maintained under a 12h light-dark cycle (lights on from 8h00 to 20h00) with *ad libitum* access to water and food. Room temperature was kept at 23 ± 1°C.

Surgical Procedure

Anaesthesia was induced by an intraperitoneal injection of a cocktail of ketamine : xylazine (100 : 10 mg/kg) then mice were placed on a stereotaxic frame (David Kopf Instruments, USA) where they were implanted with three electroencephalogram (EEG) electrodes: one above the parietal cortex (1.5mm lateral to midline and -2.5mm posterior to Bregma) and one above the frontal cortex (1mm lateral to midline and 1.5mm anterior to Bregma) and the last one above the cerebellum (1.5mm lateral to midline and 7mm posterior to Bregma) to be used as reference. Two electromyogram (EMG) electrodes were slipped between neck muscles. A subcutaneous injection of carprofen (5 mg/kg) was administered at the end of surgery for pain caring and mice were housed in individual barrels (30cm in diameter, ViewPoint Life Sciences, France) with *ad libitum* access to food and water.

Sleep Recordings

After 7-10 days of recovery, mice were connected to a cable plugged to a rotating connector (Bilaney, Plastics One, Germany) to allow free movements during recordings and were acclimated to the recording chamber for at least 3 consecutive days (Fig. 1A). The mode of acquisition for the unipolar EEG and bipolar EMG signals is detailed in Libourel et al. [16]. Brielfy, EEG and EMG signals were amplified (MCP+, Alpha-Omega Engineering, Nazareth, Israel), filtered (bandwidth 1-100 Hz for the EEG, 10-100 Hz for the EMG), digitized (CED Micro1401, Cambridge Electronic Design, sampling rate 512 Hz) and collected with a CED interface using Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Vigilance states were scored using a 5-sec window frame according to standard criteria [9].

PS deprivation procedure

No human intervention is needed during the entire experimental procedure, from the beginning of the acclimation in the recording chamber till the end of the PS recovery. Prior to the 6h and 48h automated PS deprivation, a 48h baseline is recorded starting at 8AM (ZT0) for each mouse. After 2 days of baseline recordings, automated PS deprivation starts at 10AM (ZT2) when PS amount is high, for 6h or 48h, then mice are kept in the same environment for recovery with *ad libitum* sleep.

The PS deprivation device uses a Bayesian classifier that automatically extracts EEG and EMG features and categorizes non-overlapping 5-sec epochs into Wake, SWS or PS (see [16] for detailed information). The first 12h of light phase baseline recordings are processed offline to extract templates for each vigilance state and transfer functions for the online detection. Then the closed loop selective PS deprivation can start. Every second, incoming 5-sec epochs are analyzed by the algorithm. When PS is detected, a TTL pulse (25msec, 2cm of amplitude) lifts the barrel floor up. A pulse is sent every second until PS is no longer detected. Between pulses, the platform gently returns to its initial position thanks to gravity and the presence of an air spring to break the platform fall. It results in a brisk shake of the mouse and its brief awakening (Fig. 1B). A mean of 1.9±0.7 pulses is necessary to terminate PS.

Polysomnographic analyses were done on a 24h baseline, the full PS deprivation time and the subsequent recovery period. Twelve mice went through 6h PS deprivation followed by 4h of recovery and seventeen mice through 48h PS deprivation followed by 8h of recovery.

Corticosterone measurements

Among the experimental mice, 41 mice were divided into 4 groups of 10-11 individuals, PS control (PSC, n = 10), PS deprived (PSD, n = 10), PS rebound (PSR, n = 10) and wake stimulated controls (StW, n = 11).

PSD mice went through the PS deprivation protocol for 50h30min. PSR mice were PS deprived for 48h and allowed to recover for 2h30min. PSC mice stayed undisturbed in their barrel during the entire duration of the experiment (50h30min). StW mice went through a wake stimulation protocol (50h30min) during which 1-4 randomized TTL pulses were sent to the barrel after online automated detection of a 10-sec period of wake preceded by a 20-sec period of PS or SWS. The duration of a 2h30 recovery period was based on the Fos expression profile of the PS rebound as defined in preliminary try-outs (data not shown). Mice from all groups were then sacrificed at 12:30PM.

Under deep anaesthesia (sodium pentobarbital, Ceva Santé Animale, 150 mg/kg, ip.), a blood sample (> 500µl) was rapidly transcardially collected with a heparin syringe connected to a 25G needle from the heart left and right ventricles. Samples were immediately spun (5,000rpm during 5min at 4°C) and plasma stored at -20°C in 40µl aliquots until use. Quantitative measure of plasma level of corticosterone was assessed using an ELISA assay kit following strictly manufacturer's instructions (DetectX® Corticosterone Immunoassay kit, ARBOR ASSAYS).

Spectral analysis

Spectral analysis of EEG signals was performed using a fast Fourier transform on 5-sec artifact-free epochs. Spectra were normalized to the power over the entire frequency range (0.5–20Hz) and the mean power spectrum density was calculated for SWS and PS for each animal. EEG spectral power densities were also computed across the delta [0.5–4Hz] and theta [6.5–9Hz] frequency bands.

Power densities were calculated from SWS (frontal EEG) or PS (parietal EEG) bouts during the 6h of automated PS deprivation and the 4h of recovery that followed, and during the corresponding time range in baseline.

Statistical analysis

Sleep quantities were analyzed using paired t-tests (6h PS deprivation and PS rebound), one-way ANOVA (48h PS deprivation) or two-way repeated measures ANOVA. Normality and sphericity were assessed using Shapiro and Mauchly tests respectively. Greenhouse-Geisser corrections were applied when sphericity was not assumed; Tukey's post hoc tests were performed otherwise. T-tests were unilateral for PS during deprivation and bilateral for wake and SWS analyses. Spectral analysis significance was evaluated using paired t-tests with Bonferroni's correction for multiple comparisons. Non-parametric Kruskal-Wallis tests were used on corticosterone level comparison. Post hoc Mann-Whitney tests Bonferroni-corrected were used to identify significant pair-wise differences when justified. For all statistical procedures, significance was set at 0.05.

RESULTS

Short-term paradoxical sleep (PS) deprivation with the automated method

Short-term PS deprivation is efficient

PS deprivation was performed by automated gentle shaking of the cage floor - which we will henceforth call “stimulation”, - from 10AM to 4PM, a time of day when PS is predominant. During these 6h of deprivation, mice (n = 12) fell into the PS state for only 1.22 ± 0.28% of the time (versus 9.64 ± 0.63% in baseline at the same time of day; $p<0.001$) (Table 1; Fig. 1C). A residual amount of PS is inevitable since PS has to be recognized for 1-2 sec before stimulation is triggered. Every attempt to enter PS was immediately defeated, ending PS bouts in less than 5 sec (Figs. 2C, D). The efficiency of PS deprivation was constant over time (Fig. 2A). The number of attempts to enter PS was similar to the number of PS episodes of the corresponding baseline (Table 1) except in the first hour where it was strongly decreased (mainly the first 30 min) and during the last hour where it had increased compared to baseline (Fig. 2B, 2-ways repeated measure ANOVA, $p=0.91$). Altogether, these observations indicate that the method is highly efficient for depriving mice of PS for short durations.

Short-term deprivation is specific to PS

No significant difference was seen in the quantity of SWS during the 6h of PS deprivation compared to baseline (58.3 ± 2.1% vs. 54.9 ± 1.3% respectively; $p=0.19$) (Table 1; Figs.1B, 3). The mean duration and the number of SWS bouts were not different from baseline (Table 1; Figs. 3B,C) and no fragmentation of SWS was observed, since the number of SWS bouts of different durations showed no significant difference between conditions (Fig. 3D). In addition, SWS power spectrum was similar

during deprivation and its corresponding baseline and amplitude of slow wave activity in the delta band was not different from values at corresponding baseline (Fig. 3E). However, frequency at peak was increased during PS deprivation (Fig. 3F) (2.79 ± 0.22Hz vs. 2.14 ± 0.17Hz in baseline; p=0.005, paired t-test). Finally, no increase or alteration was observed for wakefulness during PS deprivation (Table 1; Fig. 1B). Altogether, these data show that PS deprivation is specific to PS when performed for 6h.

Short-term deprivation challenges homeostatic regulation of PS

To evaluate the homeostatic regulation of PS with this 6h-protocol, we measured the build-up of PS propensity - defined as the tendency to enter PS - all along PS deprivation and determined if it would induce a rebound of PS that would compensate for PS loss during the following recovery period.

PS propensity was measured by reporting the number of attempts to enter PS per hour during the deprivation procedure. We then calculated the slope of the regression curve for each individual during PS deprivation and compared it to baseline. We found that PS propensity positively increased during PS deprivation in a manner that significantly differed from baseline (slope: 1.59 ± 0.27 vs. -0.72 ± 0.28 respectively; p<0.001) (Table 1; Fig. 2B) indicating that the increase in attempts to enter PS over time is not due to circadian time but rather to the need for PS as a consequence of the cumulative loss of PS.

Then, we analyzed the following recovery period from ZT8 to ZT12 corresponding to the 4h before lights-off (Table 2). We found a 2-fold increase in PS quantity after PS deprivation compared to baseline (15.47 ± 0.86% vs. 8.26 ± 0.69% respectively, p<0.001) in the first 3h of the recovery period (Fig. 4A). This PS hypersomnia was due to a significant increase in PS bouts duration (60.89 ± 4.36sec in recovery vs. 37.34 ± 3.13sec in baseline; p<0.001) (Fig. 4C,D) and in bouts number (29.92 ± 2.27 *vs*. 23.33 ± 1.92 respectively; p=0.05) (Fig. 4B). In addition, PS power spectrum was similar during the first 3h of recovery and the corresponding baseline (Fig. 4E). Frequency (p=0.12) and amplitude at the peak (p=0.1) were similar.

During the 4h of the recovery period, mice had recovered 43% of the amount of PS lost. Interestingly, PS latency, evaluated by the time duration between the end of the deprivation and the first PS episode, was very short (2.2 ± 0.9min). The increase in PS quantity was accompanied by a decrease in amount of wakefulness (51.3 ± 3.31min vs. 84.79 ± 5.94min in baseline, p=0.0003) and an increase in SWS (20% over baseline, p=0.04) that was due to an increase in the mean SWS-bouts duration (Table 2). The SWS power spectrum was unchanged compared to its corresponding baseline (Fig. 4F). However, the number of SWS episodes ending by a PS episode (evaluated by calculating the number of PS to SWS ratio) was significantly higher during the recovery period compared to the corresponding baseline (p=0.005) (Table 2).

To conclude, this new method of PS deprivation is suitable for short-term PS deprivation since it is efficient and very specific to PS with no significant modification of SWS and wake, except for the SWS power spectrum's frequency at peak that was slightly faster, likely due to the build-up of PS drive. Furthermore, it allows the challenging of homeostatic regulation of PS since it shows a

significant build-up of PS propensity during deprivation and a strong PS hypersomnia immediately following PS deprivation.

Long-term PS deprivation with the automated method

Depending on the question asked, a short PS deprivation might be insufficient. For instance, the identification of the network of PS regulation using c-Fos labeling requires a stronger PS rebound and thus, a PS deprivation of longer duration (48h in mice, 72h in rats) [9, 11, 17, 18]. Furthermore, we believed that it would be of interest to evaluate how PS is homeostatically regulated when PS is challenged for longer period of time. We thus performed automated PS deprivation on 17 additional mice for 48h and analyzed thoroughly sleep recordings during the PS deprivation period and the following 8h of recovery.

PS deprivation is efficient during the entire 48h of deprivation

The amount of residual PS during the 48h of PS deprivation was very limited (2.2 ± 0.2% vs. 6.2 ± 0.3% in baseline; p=1.5E-05) (Fig. 5A,B) representing only 4.6 ± 0.4% of total sleep time versus 12.6 ± 0.5% in baseline (p=7.2E-11) (Table 3). PS deprivation was more efficient on day 1 than day 2 due to an increasing number of attempts to enter PS during the deprivation procedure (Table 3; Fig. 5D). The duration of PS attempts was however the same during both days of deprivation (5.6 ± 0.5sec and 5.7 ± 0.3sec for day 1 and day 2 respectively, p=0.81) and largely decreased compared to baseline since stimulations were successful in immediately ending PS during the entire procedure (Table 3; Fig. 5C).

Deprivation is specific to PS

The suppression of PS was accompanied by an overall minor increase in wakefulness (55.19 ± 1.21% vs. 51.01 ± 1.05% in baseline; p=0.04) that was not significant in its daily distribution (Table 3; Fig. 5A). SWS quantity was unchanged during the 48h of PS deprivation (42.64 ± 1.19% of total time during deprivation vs. 42.81 ± 0.9% in baseline, p=0.6) (Table 3), and this at any time of day (Fig. 6A). The number of SWS bouts was however increased during the light phase of day 2, when sleep is predominant in baseline (Fig. 6B) and PS sleep propensity maximal (see below). This increase in SWS bouts number was accompanied by a decrease in SWS bouts duration (Table 3). The shortening effect was limited to the light phase of the second day of deprivation (Fig. 6C). Based on the 2-ways ANOVA for repeated measures analysis, the 48h of PS deprivation had no impact on SWS bouts fragmentation (p=0.34), although we found a clear tendency for an increase of the 26-50sec SWS bouts and a decrease of the longest (>80sec) SWS bouts during the second day of PS deprivation (Fig. 6D), possibly due to the strong propensity to enter PS at that time of day (Fig. 7A).

Forty-eight hours of PS deprivation strongly challenges homeostatic regulation of PS

Indeed, looking at the build-up of PS propensity we found that the number of attempts to enter PS increased all along the 48h of PS deprivation with higher number of attempts during the light phase than the dark phase in regards to the circadian distribution of PS during baseline (Fig. 5D).

Furthermore, the percentage of SWS bouts transitioning into PS attempts increased drastically during PS deprivation (Fig. 7) in particular during the light phase when PS is favored.

When PS deprivation was stopped, mice stayed in their recording barrel and were able to sleep *ad libitum* for the next 8h. In contrast to what we reported using small-platforms-over-water PS deprivation method at the same time of day [9], the latency to enter PS using this new method of PS deprivation was extremely short (1.48 ± 0.54min here vs. 113.1 ± 8.77min in Arthaud et al [9]). It was however not significantly different (p=0.48) from the latency measured after the short PS deprivation lasting for 6 h (2.2 ± 0.9min).

Mice showed a significant PS hypersomnia for the next 8h (74.56 ± 2.12min vs. 46.67 ± 1.98min during the corresponding baseline, p=4.2E-09) with the highest proportion of PS increase in the first 90min (Table 4; Fig. 8A). In that 90min time window, PS quantity represented of 25.4 ± 1.3% of total time (vs. 11.7 ± 0.7% in baseline, p=2.2E-08) and 29.8 ± 1.5% of total sleep time (vs. 15.1 ± 0.8% in baseline, p=2.7E-08) (Fig. 8B). This increase in PS amount was due to an increase in PS bouts number of longer mean duration (Table 4). It was also accompanied by a small but significant decrease in SWS (59.8 ± 1.8% vs. 66.1 ± 2% in baseline, p=0.008) and wakefulness (14.9 ± 1.7% vs. 22.2 ± 2.2% in baseline, p=0.01) (Table 4; Fig. 8B). However, the total amount of SWS and wakefulness in the first 3h of recovery period was unchanged compare to baseline (SWS: 56.2 ± 0.9% vs. 57.4 ± 1.2% respectively, p=0.44) (Table 4; Fig. 8D). Interestingly, 70% of SWS episodes transitioned into PS during the first 30min of the rebound compare to 36% in baseline (p=1.6E-04). The majority of SWS bouts ended in PS during the first 3h of PS rebound (Fig. 8C).

To determine whether PS rebound reflects the duration of the PS deprivation rather than the amount of PS removed, we tested whether PS bout duration could be explained by the duration of the previous PS-interval duration by performing a linear regression analysis on baseline recordings but found no correlation (R^2=0.003).

Finally, it has been proposed that the PS-interval duration would be controlled by the duration of the prior PS-bout duration [2]. Although we found such correlation in baseline recordings by linear regression analysis (R^2= 0.5498; p<0.0001), such link was not maintained during the first 3h of recovery (R^2= 0.0008; p=0.09) (Fig. 8E,F) likely due to the need to respond to the PS homeostatic pressure.

In summary, PS homeostasis was similarly challenged by 6 and 24h of PS deprivation. However, when PS deprivation lasted longer (with a greater quantity of PS removed) and PS propensity strongly increased, SWS was slightly more fragmented during the light phase of the second day of deprivation, while maintaining the total quantity of SWS unchanged. PS rebound showed a similar profile after short and long PS deprivation although with stronger “intensity” (increased total amount of PS) after longer PS deprivation than after a 6h short one, translating the homeostatic aspect of PS regulation.

Mice showed no sign of stress with automatic PS deprivation

We finally evaluated the level of stress potentially induced by the loss of PS during this automated deprivation method by measuring plasma corticosterone level using radioimmunoassay technology

in the 3 different conditions: after 50.5h of PS deprivation (248 ± 67 pg/mL), after 2.5h of PS rebound (171 ± 36 pg/mL), and in undisturbed controls (210 ± 41 pg/mL). Statistical analysis showed no difference in plasma corticosterone levels between groups (H=1.058, n=32, ddl=3, p=0.78, Kruskall-Wallis test) (Fig. 9B). Note that the level of corticosterone is very low in each of these conditions, compared to a stressful one such as 1h of restrained stress (Fig. 9B).

Since corticosterone level can be low in some chronic stressful condition of sleep deprivation [19], we also search for behavioral signs of stress such as piloerection or oily fur. We did not observe such behaviors. Furthermore, we looked at the latency to enter sleep after PS deprivation sessions using this new method of PS deprivation and found that it was extremely short (0.8 ± 0.3min) in contrast to the 104 ± 14 min we reported after PS deprivation in mice using the stressful platform-over-water method [3], suggesting further that mice were not stressed.

Since PS deprivation was performed by moving the cage floor up (ie. stimulation) when mice entered PS, we further checked whether such intervention might be perceived as stressful and cause sleep disturbances. We thus added an additional group of 11 mice (StW) and performed stimulations for 48h during wakefulness. We found that it did not affect PS (5.6 ± 0.5% in StW vs. 6.2 ± 0.3% in baseline; p=0.24), SWS (40.6 ± 1.3% vs. 42.8 ± 0.9% in baseline, p=0.17) nor wakefulness (53.9 ± 1.6% in StW vs. 51 ± 1.1% in baseline, p=0.13) (Fig. 9A) and had no effect on plasma corticosterone level (210.7 ± 37pg/mL in StW vs. 210.1 ± 41pg/mL in undisturbed controls, p=0.13) (Fig. 9B). It is also noteworthy that the latency to enter SWS after the very first shaking episode, while mice were not yet habituated to the PS deprivation protocol, was extremely short (2.87 ± 1.38min), suggesting that mice were not stressed by the stimulation. In other words, gentle shaking per se is not stressful and does not modify sleep properties. It is thus unlikely that it would bias the analyses.

Altogether, these data indicate that this new method of specifically depriving PS by gently shaking the cage floor without human intervention, induced no perceptible stress based on the used measures.

DISCUSSION

With this study, we demonstrate that PS homeostatic regulation is modulated (although no significant difference is observed in SWS and wake quantities) with a PS rebound depending on the total amount of previous PS deficit, and that in the context of recovery from specific PS deprivation, the PS-interval duration is no longer correlated to prior PS-bout duration.

We also demonstrate that the new automated method we have developed allows for a specific and efficient PS deprivation for short (6h) and longer duration (48h) and that gently shaking the cage floor, without human intervention, does not induce noticeable stress as we measured it. Wakefulness was undisturbed and SWS was unchanged during the 6h and up to 48h of PS deprivation. Only the second day of PS deprivation showed a tendency for an increase and a decrease in the number of, respectively, the shortest and the longest SWS bouts, paralleling the build-up of PS propensity while maintaining SWS quantity.

Technical considerations on the sleep deprivation methodology

Although circadian and homeostatic processes of regulation interact, they operate independently as shown in rats with lesion of the masterclock - the suprachiasmatic nuclei – in which the homeostatic compensatory response to PS deprivation is invariably present [20]. In the present study, recovery periods were programmed during the light phase when circadian drive allows for the occurrence of PS in a similar manner as during baseline recordings.

With previously reported deprivation techniques based on automated detection of PS (disk-over-water, "stand alone sleep deprivation" techniques), mice are forced to walk to escape a punishment (water, a bar or a divider) and accumulate muscular fatigue and stress. It has been one of the argument for the use of yoke-control animals (when the experimental animal is stimulated the yoke control is also stimulated independently of its state of vigilance) [10, 20]. Although we did not test the disk-over-water technique in mice, we have used the multiple-platforms-over-water and found that, similarly to the disk-over-water technique, it induces muscular fatigue and stress [9, 21]. In our current set-up, mice do not need to move or walk to remain safe and no locomotor activity was induced by the stimulation (video observations, data not shown) strongly suggesting that no muscular fatigue was induced. Therefore, instead of using a traditional yoke control group, we created a wake stimulated group of mice. The absence of induced locomotion was observed after stimulations performed during PS deprivation (when entering PS) as well as those randomly induced during wake in the wake stimulated control group. Furthermore, in contrast to yoke-control groups that were chronically sleep-restricted with high sleep fragmentation [20] rendering the comparison of homeostatically challenged PS with baseline sleep impossible, the wake-stimulated group showed no modification for any of the vigilance states compared to baseline.

We found that the amplitude of PS rebound was higher and the latency to PS considerably shorter using this deprivation technique compared to the values obtained for these parameters after using the small-platforms-over-water technology (ie. PS rebound is biphasic with intrusion of wakefulness and of lower amplitude) [3, 9]. One of the main differences we observed between these two methodologies is the level of stress that mice experienced as evaluated by several criteria such as plasma corticosterone level, general behavior (motion, aggression), quality of the fur and latency to enter sleep. While mice were under a chronic mild stress in the small-platform-over-water set-up, they had no detectable signs of stress with this newly developed automated PS-deprivation technology. However, since we did not test for additional biomarkers of stress (for instance measures of inflammation or behavioral tests such as open-field, light-dark box or elevated plus maze), we cannot rule out that a limited level of stress is perceived by the animals.

Libourel et al [16] also saw a highly efficient PS deprivation with a very short latency to enter PS using the same automated device in rats. They found however that the PS rebound was of lower amplitude with this method compared to the small-platform-over-water method [16]. As PS deprivation lasted for 72h in rats, PS deprivation was less specific to PS, with a significant loss of SWS in the last day. Although PS was recovered, it is likely that SWS had to be recovered too, thus competing with PS recovery. Based on our current data in mice and those obtained in rats, 48h of PS deprivation is probably the longest duration that should be used in rodents when the objective is to challenge PS without substantially modifying SWS and wake, due to the strong increase of PS propensity over time.

Gentle handling is also a stress-less technique to PS-deprive rodents [15]. However, due to detection and reaction times of the experimenters, the mean duration of PS episodes during PS deprivation by gentle handling is longer than with our automated system. For example, it was reported in rats a mean duration of PS episodes of 9.7 ± 0.6 sec with gentle handling while we found here a mean PS-bout duration of less than 5 sec. Furthermore, in contrast to our technology, gentle handling methodology relies on subjective recognition of PS and the constant attention of the experimenter during the deprivation procedure, possibly introducing bias.

Physiological considerations

During both recovery periods – following short or long PS deprivation - total PS amounts were greatly increased, in higher proportion when PS deprivation lasted longer. We found that the tendency to increase of PS propensity over time in the first 6h of PS deprivation was not yet significant. However, transitions from SWS to PS were highly favored during the following recovery period showing that specific PS deprivation as short as 6h was sufficient to challenge homeostatic regulation. When PS deprivation was pursued for longer periods of time, the number of PS bouts significantly increased during deprivation and rebound compared to baseline and the transition rate from SWS to PS was even larger (84% increase compared to the baseline).

We also found in this study that PS can be homeostatically regulated and promoted although SWS quantity (total amount and bouts duration) and quality (ie. amplitude of the slow wave activity) were unchanged. It goes against the proposal by Benington and Heller [7] which states that PS pressure accumulates during SWS, based on analyses of baseline recordings, thus without homeostatic challenges. Franken et al [8] claimed that PS homeostasis depends on wake (long-term regulation) and SWS (short-term regulation) durations based on experiments of total sleep deprivation. Although it is informative on how recovery of both sleep states compete when they are both in debt, it might have masked some specific properties of each one of them. With our automated technique of PS deprivation, PS ended by the induction of an awakening of the animal. These short arousals being of short in duration, we found no significant alteration of the SWS/Wake architecture, suggesting that PS rebound reflects essentially the amount of PS lost. We cannot however completely exclude that these short awakenings might play a role in the expression of the PS rebound. Furthermore, to test whether PS drive could accumulate (rather than being maintained to some extent) during the time elapsed from the previous PS, we evaluated whether PS bout duration was explained by the duration of the previous PS-interval duration by performing a linear regression analysis on baseline recordings but found no correlation. Altogether, our data suggest that PS rebound compensates for the amount of PS lost, independently of the duration of the prior PS-interval.

In agreement with our baseline analysis, it has been proposed that the PS ultradian regulation (ie. duration of PS-interval) would be modulated by the duration of the previous PS episode suggesting that PS pressure would be partially dissipated by each PS episode [2, 8]. Although we confirmed such correlation in baseline, our regression analysis showed that this correlation was no longer maintained in PS recovery conditions. In the context of a PS deficit, the regulatory strategy that takes place to compensate for the deficit in PS seems to vary in order to optimize its recovery. Indeed, Shea et al [22] found no change in SWS during rebound after 2h of PS deprivation. When PS

deprivation lasted 6h, we found however that the mean SWS bouts duration during rebound was increased but neither the number of SWS bouts nor the amplitude of slow wave activity were, compared to the corresponding baseline. When PS deprivation lasted even longer we found that SWS bouts duration was unchanged compared to baseline while the numbers of SWS and wake bouts were decreased and the transition from SWS to PS strongly increased.

To conclude, altogether our data indicate that the need to recover from PS deficit is dealt with by favoring transitions from SWS to PS and maintaining PS. The first strategy used to compensate for PS deficit is to increase the mean PS bouts duration without changing the number of occurrences of PS, but when insufficient, multiplying the entrance into PS is also favored. This strategy would be done at the expense of both SWS and wake, both having less opportunity to occur but when entered into, both states were stabilized.

The underlying mechanisms involved in such homeostatic regulation still need to be explored. A large body of evidence using complementary approaches show that GABAergic PS-off neurons of the vlPAG are gating PS [23-28] and when activated during PS, they favor the exit from PS into wake [2]. Determining the neuronal population modulating their activity in the context of a PS homeostatic challenge is thus crucial. Interestingly, this latter study also showed, based on recordings in baseline condition, that when the firing rate of PS-off GABAergic neurons of the vlPAG is sufficiently low, the probability of transitioning into PS is greatly increased. Future experiments aiming at recording their firing activity while mice are subjected to our new technique of highly specific PS deprivation and its following undisturbed recovery period would be of great interest in order to further determine their role in PS homeostatic regulation.

Acknowledgements:

The authors thank Dr. Christophe Varin for his precious advice on statistical analyses and Dr. Luc Gentet for English language revision. They also thank the CNRS and University Lyon1 for their financial support.

Authors' contributions: SA, PAL and CP designed the experiment. SA and PAL adapted the automated deprivation set-up to mice. SA performed the sleep experiments. CP performed corticosterone measurements. SA and CP performed analyses. CP wrote the manuscript.

Disclosure statements:

Financial disclosure : PAL is consultant at Viewpoint (France)

Non-financial disclosure : Authors have no conflict of interest.

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LEGENDS

Table 1: Data for each vigilance state (PS, SWS and wake) during the 6h of PS deprivation.

PS/SWS is the ratio of the number of PS bouts over the number of SWS bouts. It reflects the number of SWS bouts that transition into PS. Note that "slope" represents the mean value of the slope of the linear regression analysis showing a positive causal relationship between the duration of PS deprivation and the number of PS attempts. Paired-sample t-tests, ***, p<0.001. ns, non significant; TST, total sleep time.

Table 2: Data for each vigilance state (PS, SWS and wake) for the 3 first hours of recovery period (ZT8-ZT11) following the short-term PS deprivation (ZT2-ZT8). PS/SWS reflects the number of SWS bouts that transition into PS. Paired-sample t-tests, *, p<0.05; **, p<0.01; ***, p<0.001. ns, non significant; TST, total sleep time.

Table 3: Data for each vigilance state (PS, SWS and wake) when PS deprivation lasts for 48h. Statistical test: One-way ANOVA with Tukey's post hoc test; *, p<0.05; **, p<0.01; ***, p<0.001. ns, non significant; TST, total sleep time.

Table 4: Data for each vigilance state (PS, SWS and wake) during the first 90min (ZT2-ZT3.5) and 8h (ZT2 to ZT10) of recovery following long-term PS deprivation of 48h. Paired-sample t-tests, *, p<0.05; **, p<0.01; ***, p<0.001. ns, non significant; TST, total sleep time.

Figure 1: A- Photograph showing mouse environment during the entire experiment (baseline, deprivation and recovery). **B-** Graph reporting the amount of each vigilance state (mean percentage ± sem) during the 6h of PS deprivation (red) and the corresponding baseline (blue) (n = 12). Paired-sample t-tests, ***, p<0.001.

Figure 2: The efficiency of short (6h) PS deprivation. Graphs illustrating the quantity of residual amount of PS per hour (**A**) and the number of attempts to enter PS (**B**) during PS deprivation (red) vs. baseline (blue). PS attempts to enter PS were efficiently counteracted by stimulation during deprivation (red) since bouts duration lasted for less than 5sec during deprivation (**D**) at all time points (**C**) in contrast to baseline (blue). Two-way rmANOVA, p=0.91 (B), p<0.001 (A, C, D); Tukey's post hoc test BL vs. PSD ***, p<0.001.

Figure 3: The specificity of short PS deprivation. Graphs showing that total amount of SWS (**A**), and SWS bouts number (**B**) and mean duration (**C**) are not modified by PS deprivation using this new method of PS deprivation. Note that the proportion of SWS bouts of different durations is also

unchanged by deprivation (**D**). Two-way rmANOVA, p>0.05 (A, B, C, D). **E-** The global profile and amplitude of the frontal EEG power density spectrum of SWS during PS deprivation (red) are similar to those of the corresponding baseline (0-20 Hz), but the frequency at the peak is faster (**F**). Paired-sample t-test **, p<0.01.

Figure 4: Short PS deprivation induces PS hypersomnia (rebound) during the following 4h recovery period (ZT8-ZT12). **A-** Graph showing the amount of PS per hour (mean ± sem) following PS deprivation (green) compared to the corresponding baseline (blue). Note that the rebound lasts for 3h. Although the number of PS bouts is unchanged (**B**), the mean duration of the PS episodes is largely increased during the first 3h (**C**). **D-** The proportion of PS bouts of shorter duration is decreased at the benefit of longer bouts duration (31-90 and >90 sec) during the recovery period compared to baseline. Two-way rmANOVA, p=0.16 (B), p<0.001 (A, C, D); Tukey's post hoc test BL vs. PSR *, p<0.05; **, p<0.01; ***, p<0.001. The profile and amplitude of the PS parietal EEG power spectrum (**E**) and SWS frontal EEG power spectrum (**F**) of the first 3h of recovery period (green) and its corresponding baseline (blue) are unchanged. The absence of change in spectral density is illustrated normalized to baseline (black dotted line) in the inner boxes of **E** and **F**.

Figure 5: Long-term PS deprivation is efficient during the entire 48h period of PS deprivation. **A-** Histogram reporting daily quantities for each vigilance state (mean of percentage ± sem) (n = 17, One-way ANOVA, p<0.001). **B, D -** Graphs illustrating the quantity of residual amount of PS (B) and the number of attempts to enter PS (D) per hour during day 1 (orange) and day 2 (yellow) of PS deprivation vs. one day of baseline (blue). **C-** PS attempts to enter PS were efficiently counteracted by stimulation during PS deprivation since bouts duration lasted less than 5sec during PS deprivation day 1 and day 2 in contrast to baseline. Two-way rmANOVA, BL vs. PSD d1 and d2, p<0.001 (B, C, D). Tukey's post hoc test, *, p<0.05; **, p<0.01; ***, p<0.001 comparing day 1 to baseline. $^{\#}$, p<0.05; $^{\#\#}$, p<0.01; $^{\#\#\#}$, p<0.001 comparing day 2 to baseline. °, p<0.05; °°, p<0.01; °°°, p<0.001 comparing day 1 to day 2. The thick black bar shows the dark period.

Figure 6: PS deprivation is specific to PS during long-term deprivation. **A-** No difference is seen in the hourly distribution of SWS quantity (mean ± sem) during baseline (blue) and day 1 (orange) and day 2 (yellow) of PS deprivation (Two-way rmANOVA, BL vs. PSD d1 and d2, p=0.34). **B,C -** Number (B) and mean bouts duration (C) are increased and decreased respectively during the light phase of day 2 only (Two-way rmANOVA, BL vs. PSD d1 and d2, p<0.01 (B), p<0.001 (C). **D-** Proportion of SWS bouts of different duration. Long-term PS deprivation does not impact SWS bouts fragmentation (Two-way rmANOVA, BL vs. PSD d1 and d2, p=0.31).

Tukey's post hoc test, *, p<0.05 comparing day 1 to baseline. $^{\#}$, p<0.05; $^{\#\#}$, p<0.01; $^{\#\#\#}$, p<0.001 comparing day 2 to baseline. °, p<0.05; °°, p<0.01; °°°, p<0.001 comparing day 1 to day 2. The thick black bars show the dark period.

Figure 7: PS propensity increases strongly during the 48h of PS deprivation. **A-** Graph reporting the hourly evolution of the ratio of the number of PS attempts over the number of SWS bouts during the 48h of PS deprivation. Note that it increases over time but follows the circadian distribution of PS seen in baseline (blue line). Two-way rmANOVA, $p<0.001$. Tukey's post hoc test, PS deprivation vs. baseline *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. **B-** Histogram reporting the same ratio during light and dark phases of the 3 days of recordings (baseline, blue; day 1, orange; day 2, yellow). One-way ANOVA, $p<0.001$. Tukey's post hoc test ***, $p<0.001$ comparing day 1 to baseline. $^{###}$, $p<0.001$ comparing day 2 to baseline. °°, $p<0.01$; °°°, $p<0.001$ comparing day 1 to day 2.

Figure 8: Long-term PS deprivation induces a strong PS rebound during the following 8h of recovery. **A-** Mean amount of PS in 30min window frames during recovery (green) compared to the corresponding baseline (blue) during the 8h of recovery. **C-** The transition from SWS to PS is favored during the first 6h of recovery (green) as illustrated by a significantly increased PS/SWS ratio compared to baseline (blue). Two-way rm ANOVA, $p<0.001$ (A and B); Tukey's post hoc test. **B and D-** Histograms reporting the quantity of each vigilance state in the first 90min (C) and 3h (D) of the recovery period. Paired-sample t-test (C and D). PSR vs. baseline, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. **E and F-** Dot plots and linear regression analysis illustrating the absence of correlation between a PS bout duration and the duration of the following PS-interval during the first 3h of PS rebound ($p=0.09$) (F), correlation that is found highly significant during the corresponding baseline ($p=0.0001$) (E).

Figure 9: **A-** Histogram reporting the distribution of each vigilance state over 48h of stimulations during wakefulness (light blue) compared to baseline (dark blue). Stimulations during wake did not disturb sleep (n = 11, Student t-test). **B-** Histogram reporting mean value of plasma corticosterone in control undisturbed condition (dark blue), after PS deprivation (yellow), PS rebound (green) or stimulation during wakefulness (light blue). Note that PS deprivation with the automated method does not induce stress as evaluated by statistical analysis between these 4 conditions. Measures of corticosterone level were also performed in 2 mice after 1h of restrained stress (red) to validate the measurements methodology.

Table 1	Paradoxical sleep						Slow-wave sleep			Wake			
	Duration (min/6h)	**Bouts Nber**	**Bouts duration (s)**	**%/TST**	**PS/SWS Bouts**	**Slope**	**Duration (min/6h)**	**Bouts Nber**	**Bouts duration (s)**	**Duration (min/6h)**	**Bouts Nber**	**Bouts duration (s)**	**Interval between Bout (s)**
Baseline	**34.7**	**47.58**	**44.4**	**14.89**	**0.28**	**-0.72**	**197.73**	**175.5**	**70.2**	**127.63**	**176,25**	**45.11**	**81.14**
	+/- 2.3	+/- 3.7	+/- 2.4	+/- 0.8	+/- 0.0	+/- 0.3	+/- 4.6	+/- 9.1	+/- 3.6	+/- 5.2	+/- 9.2	+/- 3.4	+/- 4.1
PS Deprivation	**4.43**	**50.08**	**5**	**2.01**	**0.24**	**1.59**	**209.7**	**190.0**	**70.8**	**145.95**	**190.33**	**49.68**	**71.71**
	+/- 1.0	+/- 11.6	+/- 0.0	+/- 0.4	+/- 0.0	+/- 0.3	+/- 7.5	+/- 14.3	+/- 6.0	+/- 7.8	+/- 14.3	+/- 5.2	+/- 6.0
t value	-16.89	0.22	-17.92	-18.89	-0.93	6.92	1.38	0.88	0.14	2.04	0.85	0.75	1,33
DF	11	11	11	11	11	11	11	11	11	11	11	11	11
p value	**<0.0001**	**0.83**	**<0.0001**	**<0.0001**	**0.37**	**<0.0001**	**0.19**	**0.4**	**0.89**	**0.07**	**0.41**	**0.47**	**0.21**
	***	ns	***	***	ns	***	ns	ns	ns	ns	ns	ns	ns

Table 2	Paradoxical sleep					Slow-wave sleep			Wake			
	Duration (min/3h)	**Bouts Nber**	**Bouts duration (s)**	**%/TST**	**PS/SWS Bouts**	**Duration (min/3h)**	**Bouts Nber**	**Bouts duration (s)**	**Duration (min/3h)**	**Bouts Nber**	**Bouts duration (s)**	**Interval between Bout (s)**
Baseline	**14.87**	**23.33**	**37.34**	**15.94**	**0.32**	**80.34**	**74.42**	**65.32**	**84.79**	**73.08**	**72.9**	**84.15**
	+/- 1.2	+/-1.9	+/- 3.1	+/- 1.1	+/- 0.0	+/- 4.6	+/- 3.6	+/- 3.3	+/- 5.9	+/- 6.4	+/- 8.5	+/- 8.3
PS Rebound	**27.85**	**29.92**	**60.89**	**21.75**	**0.43**	**100.85**	**72.25**	**92.69**	**51.30**	**75.5**	**45.8**	**105.55**
	+/- 1.6	+/- 2.3	+/- 4.4	+/- 1.2	+/- 0.0	+/- 2.9	+/- 5.4	+/- 8.5	+/- 3.3	+/- 4.3	+/- 4.0	+/- 5.9
t value	-6.76	-2.19	-4.07	-7.18	-3.46	-3.7	0.29	-2.5	5.16	0.32	3.25	1.96
DF	11	11	11	11	11	11	11	11	11	11	11	11
p value	**<0.0001**	**0.05**	**0.0009**	**<0.0001**	**0.005**	**0.04**	**0.78**	**0.03**	**0.0003**	**0.76**	**0.008**	**0.07**
	***	*	***	***	**	*	ns	*	***	ns	**	ns

Table 3	Paradoxical sleep				Slow-wave sleep			Wake			
	Duration (min/24 h)	Bouts Nber	Bouts duration (s)	% / TST	Duration (min/ 24 h)	Bouts Nber	Bouts duration (s)	Duration (min / 24 h)	Bouts Nber	Bouts duration (s)	Interval between Bout (s)
Baseline 24h	**89.15** +/- 4.3	**126.71** +/- 7.4	**43.08** +/- 1.5	**12.63** +/- 0.5	**615.75** +/- 13.3	**508.12** +/- 20.9	**74.53** +/- 3.2	**733.99** +/- 15.2	**506.35** +/- 20.7	**89.94** +/- 5.3	**85.71** +/- 3.4
PS Deprivation Day 1	**21.97** +/- 3.0	**242.94** +/- 36.8	**5.60** +/- 0.2	**3.39** +/- 0.5	**630.72** +/- 27.1	**582.65** +/- 32.8	**68.17** +/- 4.5	**787.37** +/- 27.5	**583.18** +/- 33.0	**87.01** +/- 7.2	**70.48** +/- 4.5
PS Deprivation Day 2	**36.61** +/- 3.5	**403.10** +/- 43.6	**5.71** +/- 0.3	**5.77** +/- 0.6	**597.20** +/- 12.2	**679.60** +/- 42.9	**56.22** +/- 3.7	**802.30** +/- 12.3	**677.9** +/- 43.0	**76.17** +/- 5.9	**59.86** +/- 3.9
ANOVA F(2,32)	125.22	20.49	676.46	124.94	0.77	8.95	8.97	3.18	8.91	2.02	17.88
p-value	**<0.0001 *****	**<0.0001 *****	**<0.0001 *****	**<0.0001 *****	**0.47 ns**	**0.002 ****	**0.0008 *****	**0.06 ns**	**0.002 ****	**0.15 ns**	**<0,0001 *****
Tukey's post hoc test											
t-value	21.68	3.79	45.48	21.91		2.59	2.41		3.32		4.65
p-value	**<0.0001**	**0,03**	**<0.0001**	**<0.0001**		**0.17**	**0.22**		**0.08**		**0.012**
(BL *vs.* PSD d1)	*******	*****	*******	*******		**ns**	**ns**		ns		*
t-value	15.66	9.02	44.60	14.82		5.97	5.96		6.43		8.77
p-value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**		**0.0005**	**0.0006**		**0.0008**		**<0.0001**
(BL *vs.* PSD d2)	*******	*******	*******	*******		*******	*******		***		***
t-value	6.02	5.22	0.87	7.08		3.37	3.55		2.69		3.55
p-value	**0.0005**	**0.002**	**0.81**	**<0.0001**		**0.06**	**0.04**		**0.17**		**0.057**
(PSD d1 *vs.* PSD d2)	*******	******	**ns**	*******		**ns**	*		ns		ns

Table 4	Paradoxical sleep					Slow-wave sleep			Wake			
	Duration (min)	Bouts Nber	Bouts duration (s)	%/TST	Ratio PS/SWS Bouts	Duration (min)	Bouts Nber	Bouts duration (s)	Duration (min)	Bouts Nber	Bouts duration (s)	Interval between Bouts (s)
Baseline 90'	**10.60** +/- 0.6	**14.60** +/-1.0	**49.20** +/- 3.3	**15.10** +/- 0.8	**0.34** +/- 0.0	**59.50** +/- 1.8	**43.60** +/- 2.8	**88.30** +/- 6.8	**19.9** +/- 2.0	**43.12** +/- 2,.6	**31.03** +/- 5.0	**99.49** +/- 5.3
PS Rebound 90'	**22.80** +/- 1.2	**22.20** +/- 2.2	**71.70** +/- 0.6	**29.80** +/- 1.5	**0.62** +/- 0.0	**53.84** +/- 1.6	**34.90** +/- 1.9	**102.2** +/- 7.1	**13.36** +/- 1.5	**33.24** +/- 2.1	**23.35** +/- 2.6	**149.18** +/- 10.8
t value	9.66	3.2	3.85	10.02	-5.71	-3.04	-2.84	1.94	-2.88	3.75	1.46	4.59
DF	16	16	16	16	16	16	16	16	16	1616	16	16
p value	**<0.0001** ***	**0,006** **	**0,0014** **	**<0.0001** ***	**<0.0001** ***	**0,008** **	**0.012** *	**0.07** ns	**0.011** *	**0,002** **	**0.163** ns	**0.0003** ***
Baseline 3h	**19.20** +/- 0.6	**25.05** +/- 1.5	**47.66** +/- 2.1	**14.96** +/- 0.7	**0.31** +/- 0.0	**110.4** +/- 2.9	**84.35** +/- 4.6	**82.59** +/- 5.3	**50.36** +/- 2.7	**84.59** +/- 4.5	**37.53** +/- 2.9	**95.97** +/- 5.6
PS Rebound 3h	**35.60** +/-1.4	**34.82** +/- 2.7	**65.06** +/- 3.4	**25.79** +/- 1.0	**0.57** +/- 0.0	**103.6** +/- 3.5	**61.71** +/- 3.5	**104.1** +/-5.2	**41.06** +/- 3.8	**60.24** +/- 3.6	**44.91** +/- 7.5	**143.94** +/- 6.5
t value	11.67	3.52	4.46	9.69	7.13	-1.23	-4.31	3.99	-1.92	4.86	0.83	7.45
DF	16	16	16	16	16	16	16	16	16	16	16	16
p value	**<0.0001** ***	**0.003** **	**0.0004** ***	**<0.0001** ***	**<0.0001** ***	**0.23** ns	**0,0005** ***	**0.001** **	**0.07** ns	**0.0002** ***	**0.41** ns	**<0,0001** ***

Figure 1

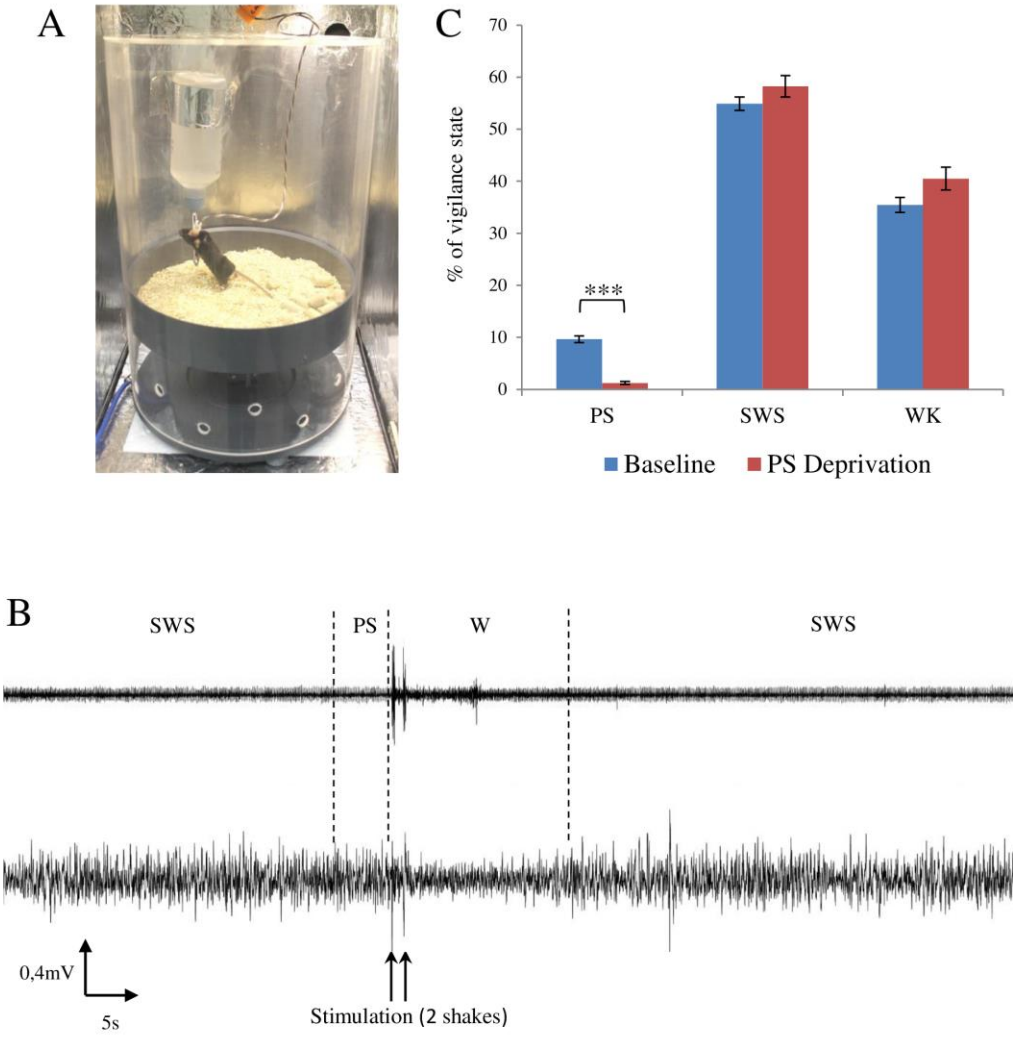

Figure 2

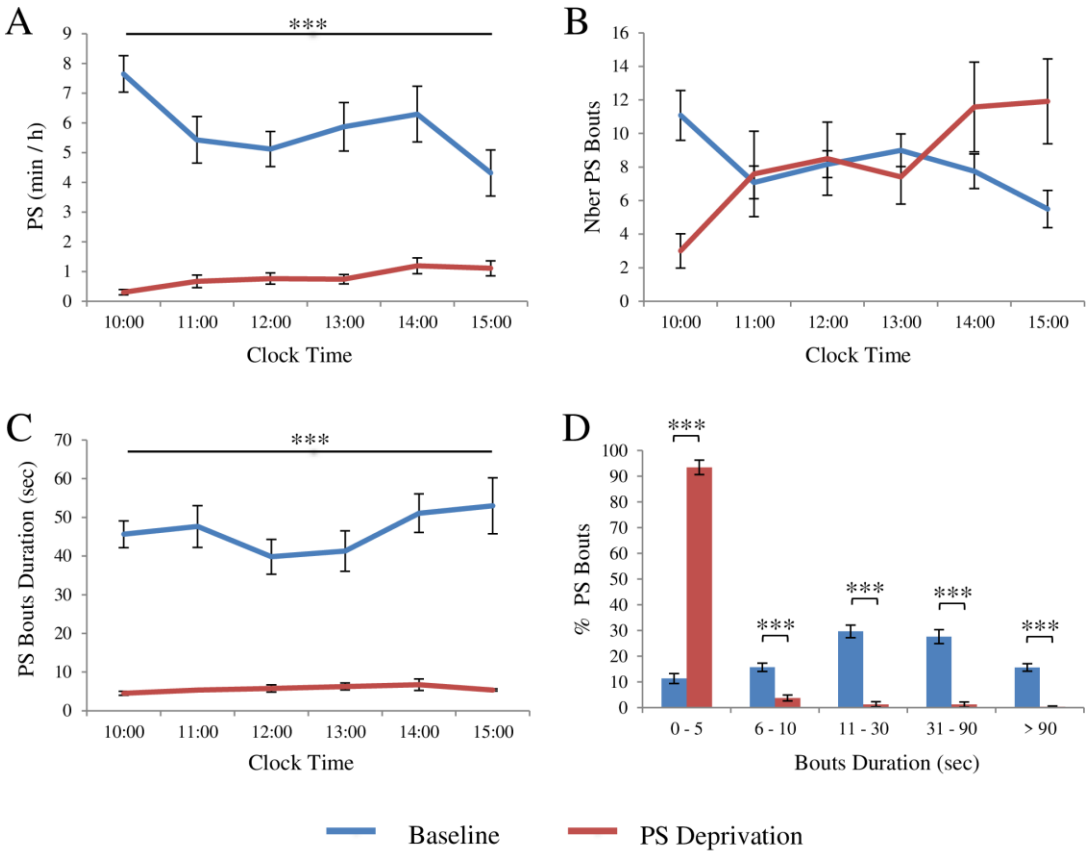

Figure 3

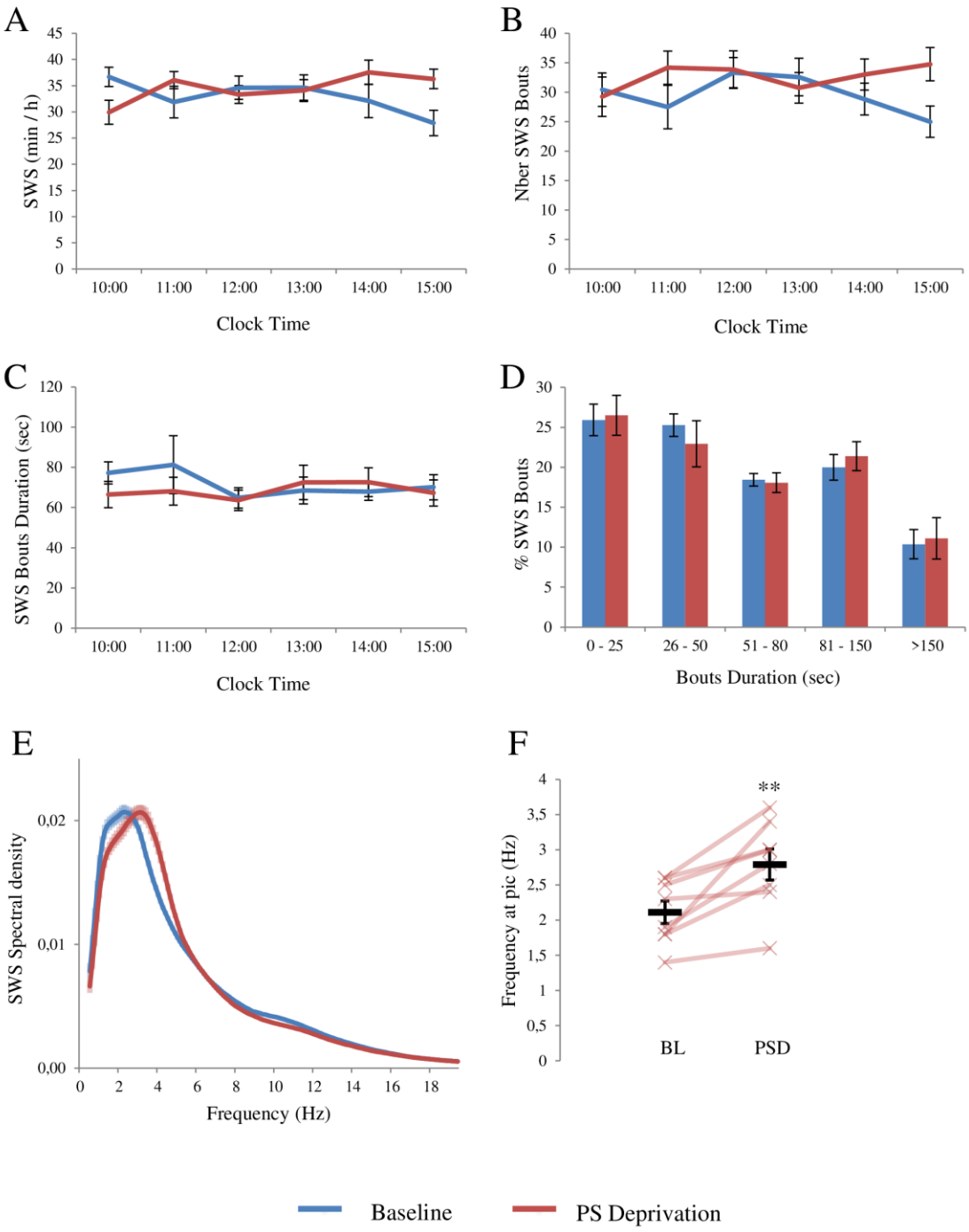

Figure 4

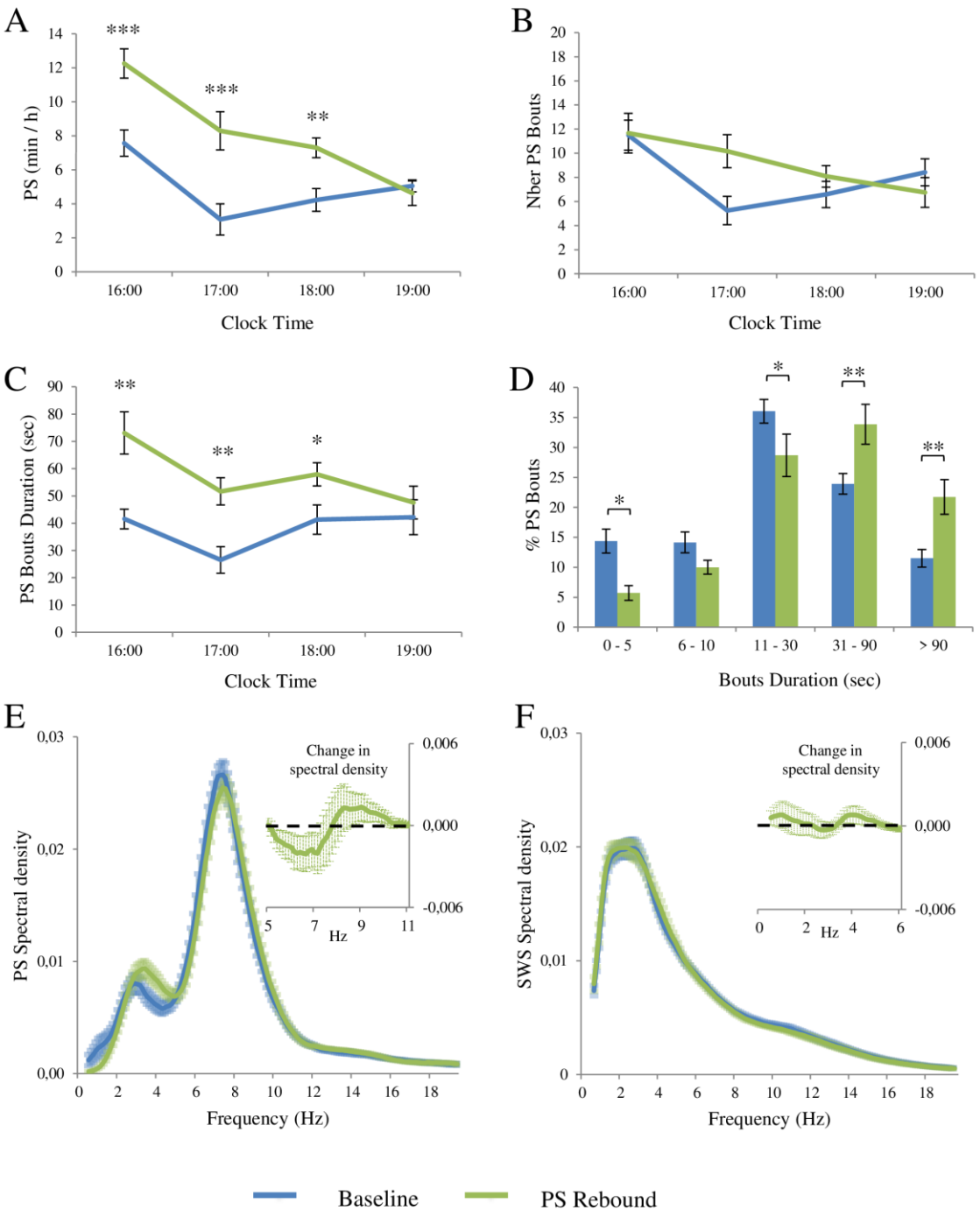

Figure 5

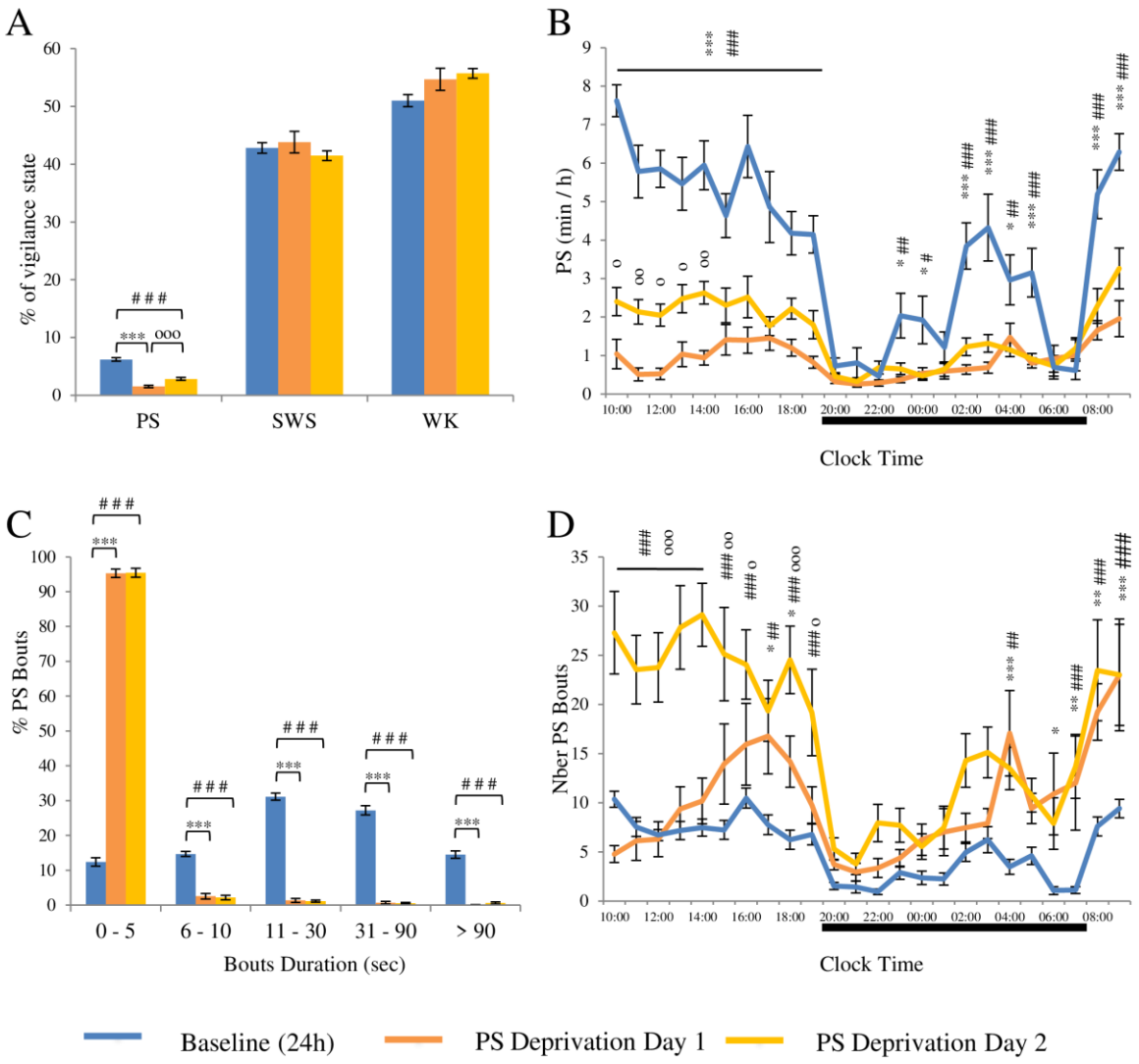

Figure 6

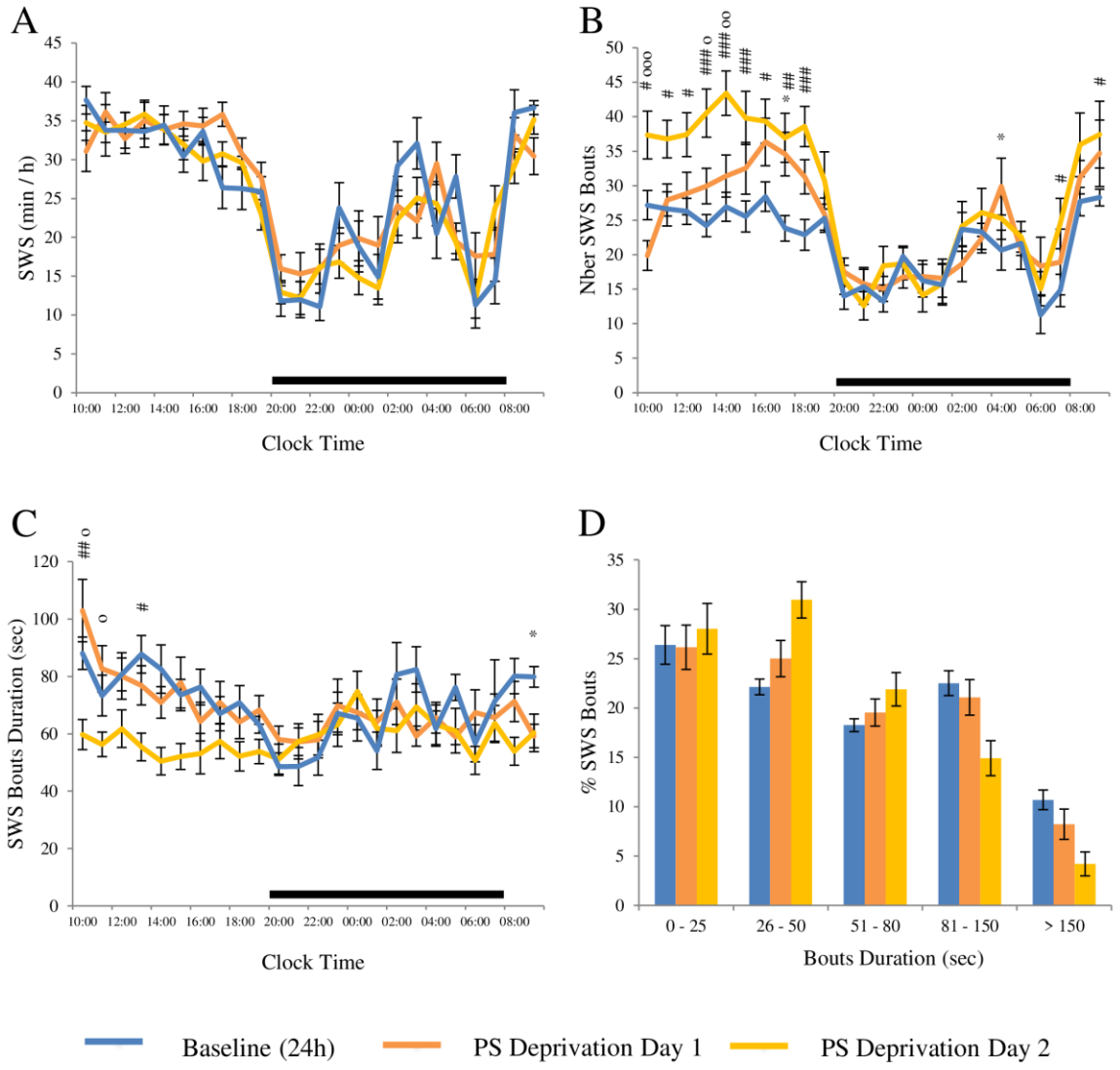

Figure 7

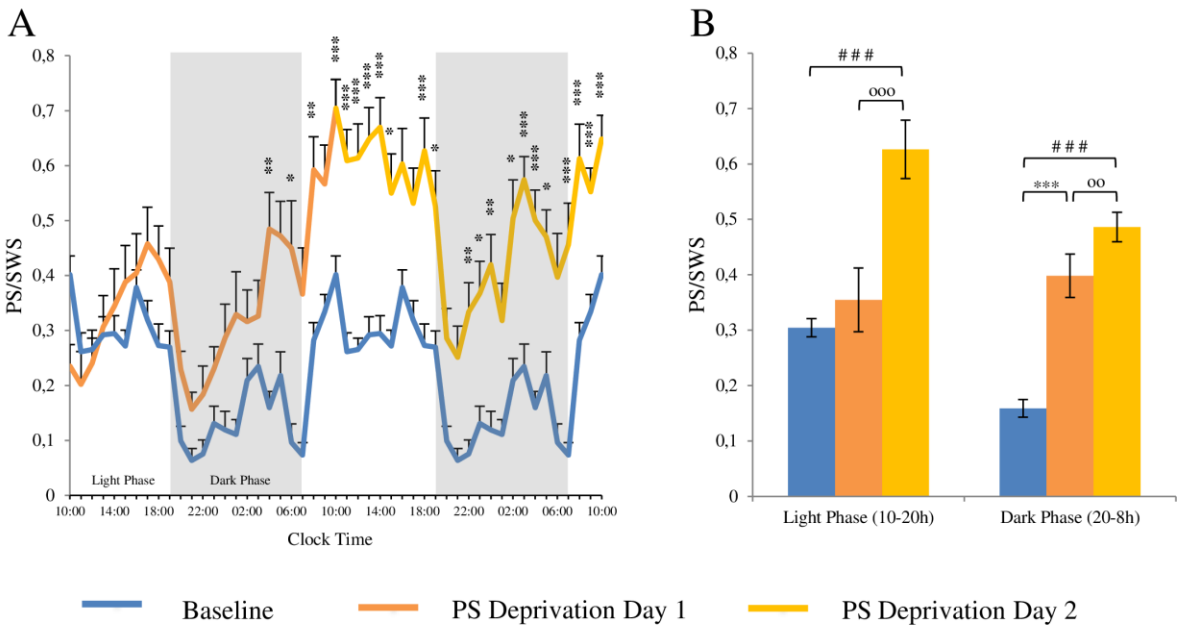

Figure 8

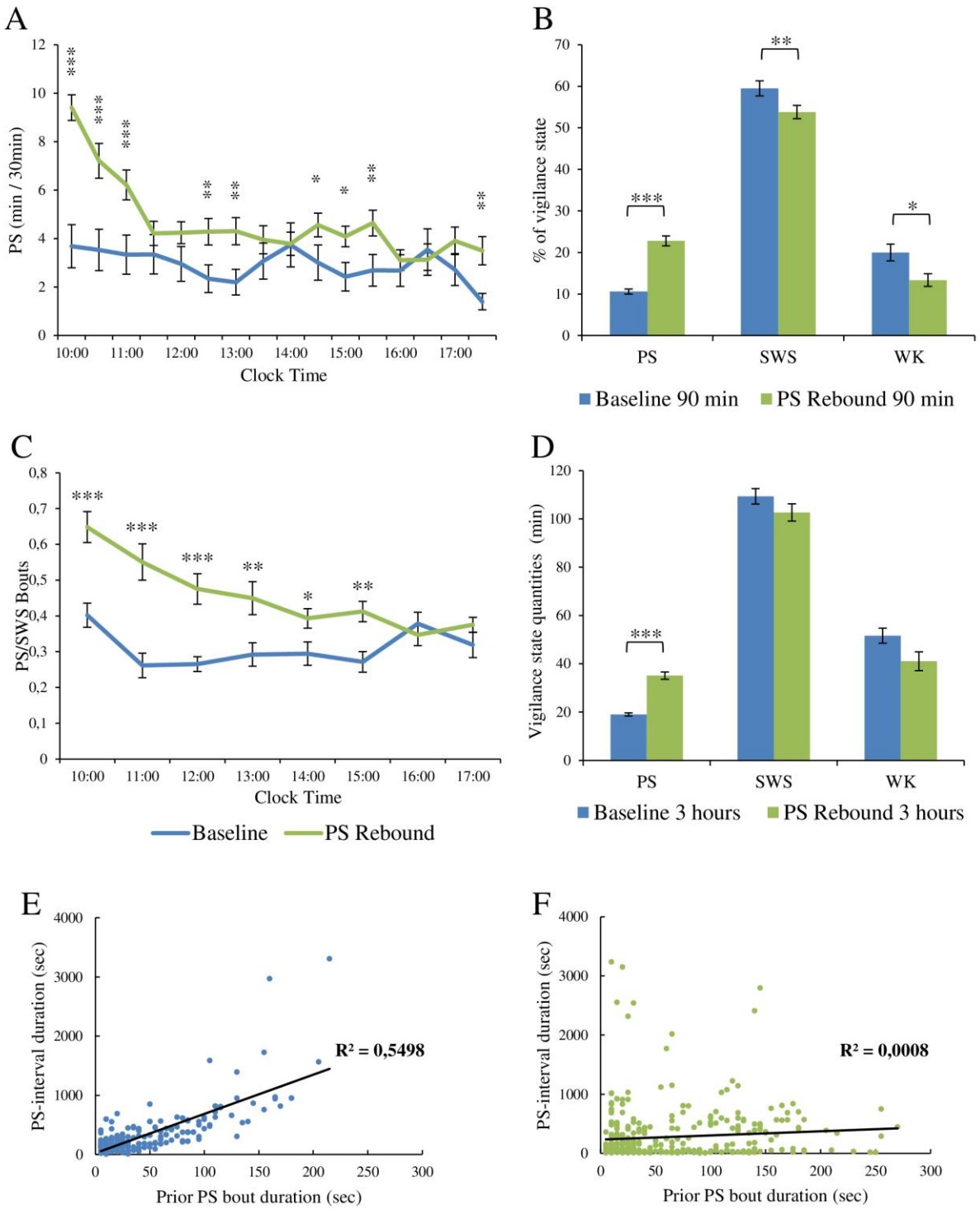

Figure 9