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Persistence of neural function in animals submitted to seizure-suppressing scale-free nonperiodic electrical stimulation applied to the amygdala

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ABSTRACT

Based on the rationale that neural hypersynchronization underlies epileptic phenomena, nonperiodic stimulation (NPS) was designed and successfully tested as an electrical stimulus with robust anticonvulsant action. Considering the scale-free temporal structure of NPS mimics natural-like activity, here we hypothesized its application to the amygdala would induce minor to none impairment of neural function in treated animals. Wistar rats underwent gold-standard behavioral tests such as open field (OF), elevated plus-maze (EPM), novel object recognition, and social interaction test in order to evaluate the functions of base-level anxiety, motor function, episodic memory, and sociability. We also performed daily (8 days, 6 h per day) electrophysiological recordings (local field potential/LFP and electromyography) to assess global forebrain dynamics and the sleep-wake cycle architecture and integrity. All animals displayed an increased proportion of time exploring new objects, spent more time in the closed arms of the EPM and in the periphery of the OF arena, with similar numbers of crossing between quadrants and no significant changes of social behaviors. In the sleep-wake cycle electrophysiology experiments, we found no differences regarding duration and proportion of sleep stages and the number of transitions between stages. Finally, the power spectrum of LFP recordings and neurodynamics were also unaltered. We concluded that NPS did not impair neural functions evaluated and thus, it may be safe for clinical studies. Additionally, results corroborate the notion that NPS may exert an on-demand only desynchronization effect by efficiently competing with epileptiform activity for the physiological and healthy recruitment of neural circuitry. Considering the very dynamical nature of circuit activation and functional activity underlying neural function in general (including cognition, processing of emotion, memory acquisition, and sensorimotor integration) and its corruption leading to disorder, such mechanism of action may have important implications in the investigation of neuropsychological phenomena and also in the development of rehabilitation neurotechnology.

1. Introduction

Epilepsy is a serious neurological disorder that affects approximately 1% of the world population [1]. Refractoriness, afflicting circa 30% of patients [2,3], is usually devastating, given that the unpredictability of seizures largely impairs access to leisure, work, and even daily activities such as driving [4]. On top of that, only around half of the refractory patients can be safely submitted to ablative surgery [5], rendering a significant number of individuals helpless and suffering from a greatly decreased quality of life, stigma, and also the economic burden related

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to treatment and hospitalization costs, work leave, and unemployment [4].

For over a decade now, our group has been working on the development of a non-standard form of low-energy electrical stimulation (ES) to suppress seizures as a means to treat refractory epilepsy [6]. In a pioneering study, we were able to suppress seizures acutely induced by intravenous injection of the GABAergic antagonist pentylenetetrazol in rats by randomizing time intervals between ES pulses (IPI: interpulse intervals), even with a very low count (only four on average) of pulses per second (Fig. 1 A right panel) [7]. This method contrasts mainstream state-of-the-art approaches, which currently deploy fixed frequency stimuli of at least 100 Hz and up to circa 350 Hz [8]. Considering that pulse morphology (current amplitude and duration) is in levels compatible with other approaches, this form of low-frequency stimulation is able to deploy a potent antiepileptic effect with a minimum transfer of electrical charge to neural tissue (0.2 μC per second) [9]. This feature is advantageous since it implicates in a decreased risk of tissue lesion related to electrolysis, heat and other non-faradaic processes, while also extending battery longevity in the circumstance of an implantable pulse generator.

The rationale behind this new method, termed NPS (nonperiodic stimulation), is that a complex, non-regular, temporal pattern of stimulus may disrupt aberrant synchronization between neural oscillators and across neural circuitry responsible for hypersynchronous phenomena supporting ictogenesis [10]. As a non-pharmacological therapeutic alternative, it may also bypass probable causes of refractoriness putatively related to lower levels of brain organization, such as vascular, cellular, and molecular factors [11]. In fact, NPS has also been successfully tested against chronic seizures spontaneously occurring in dysfunctional hyperexcitable tissue of rats in the late phase after pilocarpine-induced status epilepticus [12] and displayed promising results when applied non-invasively to human patients (manuscript under review). Also, behavioral, electrophysiological, and imaging studies have found evidence that NPS is able to rectify signals of aberrant synchronization in treated animals [13–17]. Finally, testing of similar approaches of tempering with the conventional fixed frequency temporal pattern of ES has been carried out by different research groups as attempts to normalize cortical excitability [18], suppress ictogenesis [19], or even impair epileptogenesis [20], yielding different levels of success.

There is now accumulated evidence suggesting a network-desynchronizing effect of NPS and other similar approaches as a therapeutic mechanism against epilepsy and other disorders [21]. Furthermore, novel insights have contributed to an understanding that a complimentary mechanism may be in place in the anticonvulsant effect displayed by our method. The algorithm used to generate the temporal pattern of NPS was devised to be carried out in real-time with a random but dependent (to the last pulse) fashion, mimicking scale-free processes. In fact, the distribution of IPI in this stimulus follows a power-law of unitary exponent (Fig. 1 A left panel), which has been reported as the optimal temporal pattern for engaging single neurons [22,23] or even small neuronal networks [24] in high-fidelity firing responses in vitro. Therefore, we put forward the hypothesis that NPS is a mimetic for scale-free natural-like input and, thus, may compete with aberrant epileptiform activity for the recruitment of neural substrates and networks. In turn, this competition may underlie seizure suppressing while keeping synchronization levels close to baseline and serving as a very beneficial on-demand-only desynchronization treatment. These ideas have been more thoroughly explored elsewhere [6] and their rationale has been put forward as a hypothesis to the anticonvulsant effect of the endocannabinoid system [25].

The aspect of on-demand-only effect is of paramount importance regarding the translational application of NPS since such mechanism of action would yield minor side effects in terms of core neural functions and oscillatory activity underlying homeostasis. In the present study, we investigated this possibility by evaluating neural function and the overall integrity of the major circadian rhythm inherent to the sleep-wake-cycle in animals submitted to NPS. Analogously to previous studies by our group, the selected anatomical target was the basolateral amygdala (BLA) for several reasons: (1) alongside the hippocampus, it is seen as a substrate of major importance in the genesis of epileptic phenomena [26–28] (2) it plays a key role in the processing of information within the limbic system and in neuromodulation [29]; (3) it displays widespread connectivity with areas in the forebrain, midbrain, and hindbrain [30]. Overall, there is now enough evidence indicating that the amygdala is a major participant in the effects seen in NPS [9,14,15].

Due to its privileged location and wide connectivity with forebrain structures, the amygdala is also related to several neural functions [31], as well as psychiatric and neurological diseases [32]. In particular, together with the hippocampus, the nucleus accumbens, and the hypothalamus, it constitutes the limbic system, which is responsible for integrating sensations of external stimuli with the body's information, originating emotional experiences [33], and the formation and processing of some types of memory [32], such as aversive memory, for example [34]. The basolateral nucleus of the amygdala, specifically, is related to the production and expression of anxiety and fear [35]. In fact, a common characteristic of anxiety disorders is the hyperexcitability of this nucleus, which can lead to pathological anxiety and panic syndrome [36]. Additionally, the amygdala is also related to the modulation of locomotor activity [37], a parameter used in several behavioral anxiety tests [38]. Furthermore, the amygdala is involved in assessing the environment and verifying the existence of potential hazards in the expression of social behavior [31]. With this information in mind, the present study aimed to evaluate episodic memory, motor function, base level anxiety, and social interactions in animals undergoing NPS using the gold standard for well-established behavioral tests. Moreover, we also investigated the sleep-wake cycle architecture using video and electrophysiological (electroencephalography and electromyography) recordings over long periods as a means to assess global forebrain neurodynamics.

2. Methods

2.1. Animals and groups

All experimental procedures were previously approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of São João del-Rei (UFSJ) (Protocols 025/2015, 023/2016, and 024/2015 - addendum 17/2016) and are in accordance with international guidelines for the care of animals in research.

A total of 56 male Wistar rats weighing 250–380 g were used in this study. The animals were supplied by the UFSJ vivarium and kept in a 12-hour light-dark cycle (lights on at 7 am and off at 7 pm), at 23º ± 2ºC, with food and water ad libitum.

The rats were randomly allocated into five groups: control (CTRL, n = 14), surgical control (SHAM, n = 9), nonperiodic stimulation (NPS, n = 13), social interaction pair (CTRL/SI, n = 14), and those submitted to sleep monitoring with simultaneous NPS stimulation (SWC/NPS, n = 6).

Behavioral experiments were carried out with groups CTRL, SHAM, NPS, and CTRL/SI between 8 am and 1 pm. During behavioral tests, only animals in the NPS group were subjected to electrical stimulation (see Section 2.3 for details). CTRL animals composed the baseline for comparison, while SHAM animals (with electrode implants but without stimuli) were used to control for effects of the surgical procedure (Fig. 1D). Video monitoring and electrophysiological recording of the SWC/NPS group were performed continuously between 10 am and 4 pm during 8 consecutive days. NPS, in even days (2, 4, 6, and 8), was also applied to these animals (Fig. 1E).

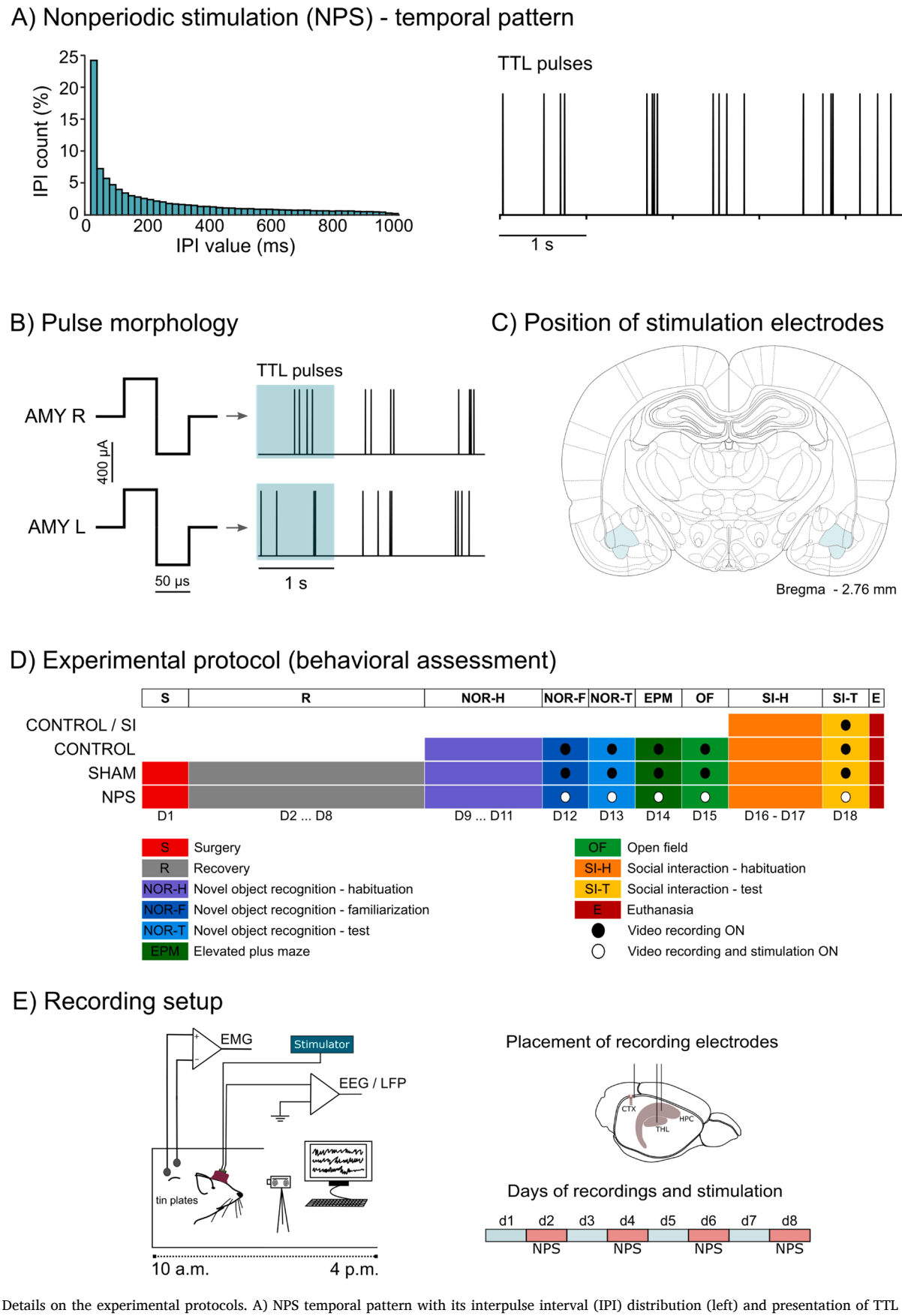

Fig. 1. Details on the experimental protocols. A) NPS temporal pattern with its interpulse interval (IPI) distribution (left) and presentation of TTL control pulses across time (right). B) Pulse morphology and asynchronous application to right and left amygdala. C) Bilateral amygdala as substrates for NPS application. D) Chronology of behavioral tests as applied to each animal group. E) Details of electrophysiological experimentation, showing a diagram of the setup (left, placement of recording electrodes, and chronology of recording and stimulation (right).

2.2. Surgical procedure

All animals, except those in the CTRL and CTRL/SI groups, underwent stereotaxic surgery for the implantation of stimulation electrodes into the BLA in both brain hemispheres. Ketamine (100 mg/Kg – Konig do Brasil, Santana do Paraíba, SP, Brazil), xylazine (5 mg/Kg – Syntec do Brasil, Cotia, SP, Brazil), and fentanyl (0.025 mg/Kg – Union Chemical do Brasil, Londrina, PR, Brazil) were used for anesthesia. The implanted bipolar electrodes (set up in twisted pairs, with a distance of 0.5 mm between the tips) were made of stainless-steel wires coated with Teflon (diameter 127 μm, model #791400, A–M Systems Inc., Sequim, WA, USA). Implantation was performed according to the coordinates provided by the Paxinos and Watson [39] neuroanatomical atlas: AP $= -2.8$ mm and ML $= \pm 5$ mm from the bregmatic suture and DV $= 7.2$ mm from the dura mater, with the aid of a stereotactic device (Insight Equipamentos Ltda., Ribeirão Preto, SP, Brazil) (Fig. 1 C). In the SWC/NPS group, two additional monopolar local field potential (LFP) recording electrodes (Teflon coated stiffened stainless-steel wires; model #7916, A–M Systems Inc., California, USA) were implanted into the thalamus (AP $= +3.0$ mm, ML $= +2.6$ mm, DV $= -6.4$ mm) and hippocampus (AP $= +2.8$ mm, ML $= +1.5$ mm, DV $= -3.3$ mm) in the right hemisphere (Fig. 1E). Also, one additional recording electrode made of a surgical self-tapping bone screw (Fine Science Tools, Inc., Canada) was visually positioned in the right parietal cortex. In order to detect the muscle tonus during different sleep-wake cycle states, two electrodes with rounded tin tips welded to stainless steel wires (model #7916, A-M Systems, Sequim, WA, USA) were inserted into the neck muscles for differential electromyographic (EMG) recording in the SWC/NPS group. EMG leads were passed under the animal's skin through the dorsal region unto the opening in the head. Recording and stimulation electrodes were fixed to the skull with zinc cement (Vigodent – Coltene Company, Rio de Janeiro, RJ, Brazil). All electrode leads were then welded to RJ-type connectors according to their number and group: RJ-11 (6 leads) in groups SHAM and NPS and RJ-45 (8 leads) connectors in group SWC/NPS. Finally, leads and connector were fixed in the animal's head using autopolymerizing acrylic (Artigos Odontológicos Clássico Ltda., São Paulo, SP, Brazil). Correct positioning of brain electrodes was later verified by histology by means of visual inspection with the aid of a magnifying glass.

2.3. Electrical stimulation

For the bilateral electrical stimulation of the amygdalae in vivo, the nonperiodic temporal pattern (NPS) was used, i.e., with random intervals between pulses and an average frequency of 4 Hz, in other words, 4 pulses per second (Fig. 1B). Pulses were delivered in the form of square, biphasic current waves, with zero net charge, 100 μs of total duration, and varying amplitudes of 50–600 μA. Bipolar electrodes were used and the pulses were delivered asynchronously between right and left hemispheres (AMY R and AMY L). The electric current amplitude was determined for each rat the day before the experiments, according to the animal's susceptibility, in order to select the value capable of generating the desired therapeutic effect but with minimal or even no effects on overall behavior. As performed in all other studies of the group, by increasing the current amplitude at 50 μA steps, stimulation current was defined as 100 μA lower than the first value capable of eliciting twitches. More details on NPS and pulse randomization can be found in Cota et al. [7].

The electrical stimulator used to stimulate the animals in the exploration and object recognition test was developed and built by our research group [15]. It consisted of a constant-voltage isolation unit activated by an MP3 player output (model NWZ-B1S2 26B, Sony), all powered by batteries. In order to monitor and calibrate the applied current, we measured the voltage across a 50 Ω shunt resistor connected in series. The nonperiodic pulse pattern (NPS) was created using the Audacity® software and later recorded on a generic MP3 player, which, in turn, was connected to the stimulator to trigger the system. An off-the-shelf stimulation device (model 3800, A-M Systems, Sequim, WA, USA), which has its own software to control the morphology of electric pulses and to fire conventional protocols of stimulation, was used for the electrical stimulation of the animals in the other behavioral tests and during LFP recordings in the experiments of forebrain neurodynamics across the sleep-wake cycle. In order to obtain the NPS stimulus, a software script developed and based on Cota et al. [7] was used to control two Arduino boards responsible for the real time generation of two independent NPS patterns. Output TTL pulses triggered the stimulation control unit (model 3800) for the configurations of phases and duration, which, in turn, was connected to the two isolation units (model 3820) for the configuration of fixed current amplitudes.

2.4. Behavioral evaluation

In order to investigate the effect of NPS on neural function, behavioral tests were conducted with the groups CTRL, CTRL/SI, SHAM, and NPS (Fig. 1D). The object exploration and recognition test evaluated memory, the elevated plus maze (EPM) and open field maze (OFM) were used to assess anxiety (the latter was also used to analyze locomotor activity), and the social interaction test was conducted to evaluate social behavior. All animals were submitted to all behavioral tests in the sequence shown in Fig. 1D, some being removed due to routine methodological complications, resulting in a different number of rats (N) in each stage of the experimental protocol. Electrical stimulation was applied to animals in the NPS group (only) simultaneously at the beginning of each behavioral test and throughout its duration, except in the habituation phase to the experimental arena. The sequence of behavioral tests lasted 10 days, and the procedures performed with each group are shown in Fig. 1D.

After seven postoperative days of recovery, animals were submitted to the experiments. The first test consisted of novel object recognition (NOR), conducted to evaluate memory. Held in a square arena, this evaluation took place over five consecutive days: three days for habituation to the arena, 30 min each day; one day of familiarization (F) to four different objects for 20 min; and one test day (T), with the exploration of four objects during 20 min, in which two of the four familiar objects (Fo) were replaced by two new ones (No) [40,41]. Time (t) spent exploring each object on days F and T was tabulated manually with the aid of the PlusMz software, developed by a collaborator and adapted by our group. Data on this parameter were normalized using the formula proposed by Mumby et al. [40]: [t new objects/ (t new objects + t familiar objects)]. Comparisons were also normalized by the day F, which led to the normalization of the dataset:

[t familiar objects/ (t new objects + t familiar objects)]·

In order to assess anxiety, the elevated plus maze (EPM) and open field (OFM) tests were performed. In the EPM test, the animal's duration of stay in the open (oa) and closed (ca) arms of the labyrinth were counted during a single 5-minute session [42,43]. The time count was conducted semi-automatically (manual-entry with automated summation), with the PlusMz open-source software, developed by a collaborator. In the OFM test, the animal's duration of stay in the outer area (OA) and center (C) of the arena were also computed during a single 5-minute session. Similarly, this test assesses locomotor activity based on the number of crossings between quadrants [43–45]. The OFM test was held the day after the EPM test, using a standard circular arena. Data was computed semi-automatically (manual-entry with automated summation) with the aid of the OpenFLD open-source software, also developed by a third-party collaborator.

The goal of the fourth and last experiment was to evaluate social behavior. To this end, the social interaction (SI) test was carried out, which consisted of two days of habituation to the arena for 15 min each day and one day of interaction with a second animal for 10 min. The

analyzed behaviors were: following, sniffing, mounting, and aggression [43,46,47]. The expression time of each behavior was computed semi-automatically (manual-entry with automated summation) with the aid of the XPloRat open-source software [48]. This assessment was conducted in the same arena used in the OFM test.

After the end of each experiment, the apparatuses were properly sanitized to perform the test with the next animal. Behavioral evaluations were filmed and watched later for data computing and tabulation to carry out statistical analyses.

2.5. Electrophysiological recordings

Animals in the SWC/NPS group were monitored by electrophysiological recording (EMG and LFP) and their behavior was filmed over the course of 8 consecutive days. On odd days (1st, 3rd, 5th, and 7th), recordings were conducted without the application of NPS; whereas on even days (*e.g.*, 2nd, 4th, 6th, and 8th), NPS was delivered throughout the entire recording period. Prior to the beginning of the experiments, animals were taken to the recording system arena at least 1 h before the actual recording. Once connected to the system, they were monitored for a period of 6 h/day (10 am to 4 pm). LFP signals were filtered at 0.3–300 Hz and amplified with a 2000 V/V gain, EMG signals were filtered at 30–300 Hz and amplified with a 1000 V/V gain, and then both signals were digitized at 1 Ksamples/second, using an off-the-shelf amplifier system (model #3500, A-M Systems, Sequim, WA, USA) connected to a 12-bit resolution analog to digital converter (PCI 6023E, National Instruments, São Paulo, SP, Brazil). Acquisition was controlled by a built-in virtual instrument using LabVIEW® and raw data series were processed using built-in Matlab routines.

2.6. Assessment of the sleep-wake cycle architecture and forebrain dynamics

In order to study the impact of NPS on the neural activity, we performed both manual and automated electrophysiological analysis. First, all recordings were visually inspected by an experienced researcher in order to identify the three periods of major states of the sleep-wake cycle (SWC): wakefulness (WK), slow-wave sleep (SWS), and rapid-eye movement sleep (REM). By using scaled visualization graphs, identification of onset and termination times of each stage was possible according to objective criteria: WK characterized by fast desynchronized LFP with high amplitude EMG; SWS characterized by slow (delta range) high-amplitude LFP and decreased EMG amplitude and; REM characterized by desynchronized LFP or theta-range quasi-sinusoidal rhythms with near-zero amplitude EMG (Fig. 6 A). After SWC staging was complete, it was possible to calculate the following parameters of interest: total wake time, total sleep (SWS+REM) time, proportion of duration for each stage, number of episodes of each stage, and number of transitions between stages.

Next, in order to assess the overall oscillatory content of the distinct SWC stages, we performed a thorough spectral analysis. First, LFP tracings from the hippocampus were computed for the frequency spectrum to obtain relative power and peak frequency for the delta (0.5–4 Hz) and theta (5–9 Hz) ranges, separated in the distinct SWC stages. These values were compared across the many days of experimentation. In the sequence, we analyzed the global forebrain dynamics by computing daily state maps of spectral content of the whole recording from all three brain areas, as described elsewhere [49]. Briefly, the spectral content of each 1-second window of LFP recording of all channels is separately computed and the ratios between two pairs of band powers are calculated: ratio 1 [(0.5–20 Hz) / (0.5–55 Hz)] and ratio 2 [(0.5–4.5 Hz) / (0.5–9 Hz)]. A principal component analysis (PCA) is thus computed over all the results and the timeseries of the two firsts principal components (PC1 and PC2) obtained are smoothed by a 20 s Hanning window. Points in the smoothed timeseries of PC1 and PC2 are then plotted in the X and Y axes of the state map, respectively. It has been shown that such depiction of long-duration LFP recordings results in clear clusters of points that can be objectively detected, and which are directed linked to each one of the three major SWC stages [49]. Moreover, while their positions and sizes remain the same in healthy animals, brain perturbations with electrophysiological correlates induce clear modifications of the state map (see for instance Dzirasa et al. [50]). In this work, we performed the automated method described by Gervasoni and colleagues [49] to detect the clusters for WK, SWS, and REM, which is based on the velocity of movement in the state space (points move slowly inside clusters and faster during transitions). By setting the velocity threshold artificially high, we were able to very sharply separate the clusters without any kind of spatial overlapping. Although this left many points unclassified, this was necessary to objectively compare the clusters along the experiment.

Finally, total daily energy of EMG, separated by SWC stages, were also calculated by simply squaring voltage values.

2.7. Statistical analyses

Parametric data were analyzed using the one-way ANOVA test and Tukey's post hoc test was used for multiple comparisons. The Kruskal-Wallis test was used for non-parametric data and multiple comparisons were performed with Dunn's post hoc test. Results are expressed as mean ± s.e.m. or median (range) for parametric and non-parametric data, respectively, and were considered statistically significant when $p < 0.05$.

2.8. Histology

At the end of the experiments, all animals were euthanized with an anesthetic overdose of isoflurane gas. The brains of animals from groups that received electrode implants were extracted and preserved in 10% formaldehyde for later histological processing. Brain sections were made using a freezing microtome. The coronal sections were 50 μm thick, placed on gelatinized slides, and later stained in violet cresyl (Nissl stain). In order to confirm the position of the electrodes, the sections were analyzed using a stereoscopic magnifying glass. It was confirmed that all the electrodes correctly reached the targeted areas.

3. Results

In the first behavioral test (NOR), significant differences were identified in all groups on day T, and new objects were explored in a higher proportion than the familiar ones: CTRL: * ** *$p < 0.0001$; SHAM: *$p < 0.05$; NPS: * ** *$p < 0.0001$ (Fig. 2).

In general, no significant differences were observed between groups in any of the other studied behaviors, as it can be seen for the EPM (Fig. 3; notice that O/C data is depicted as box plots), OFM (Fig. 4), and social interaction (Fig. 5) tests. There were two exceptions: a smaller number of crossings in the outer area of the OFM by the SHAM group (vs. CTRL; **$p < 0.01$) (Fig. 4, top-right panel) and a longer period of expression of the "following" behavior by the SHAM group (vs. NPS; *$p < 0.05$) (Fig. 5,bottom-right).

Visual inspection of electrophysiological recordings revealed clear LFP and EMG signatures characteristic of each stage of the SWC, as previously described (Section 2.6). There was no observable difference among recordings across days of experiment. As displayed by an illustrative case in Fig. 6 A, SWC stages can be clearly identified and there is no overt modification between the first day (top 6 tracings) and the last day (bottom 6 traces). Distribution of time spent awake or asleep did not changed significantly along the 8 days of experiment, as indicated by values of total awake time normalized by total sleep time (TST) (Fig. 6B left) and absolute TST (Fig. 6B right). When stages are separated individually, there are also no differences in proportion of time (Fig. 7 A), number of episodes for each stage (Fig. 7B left column), time spent in each stage (Fig. 7B center column), and mean duration of each episode

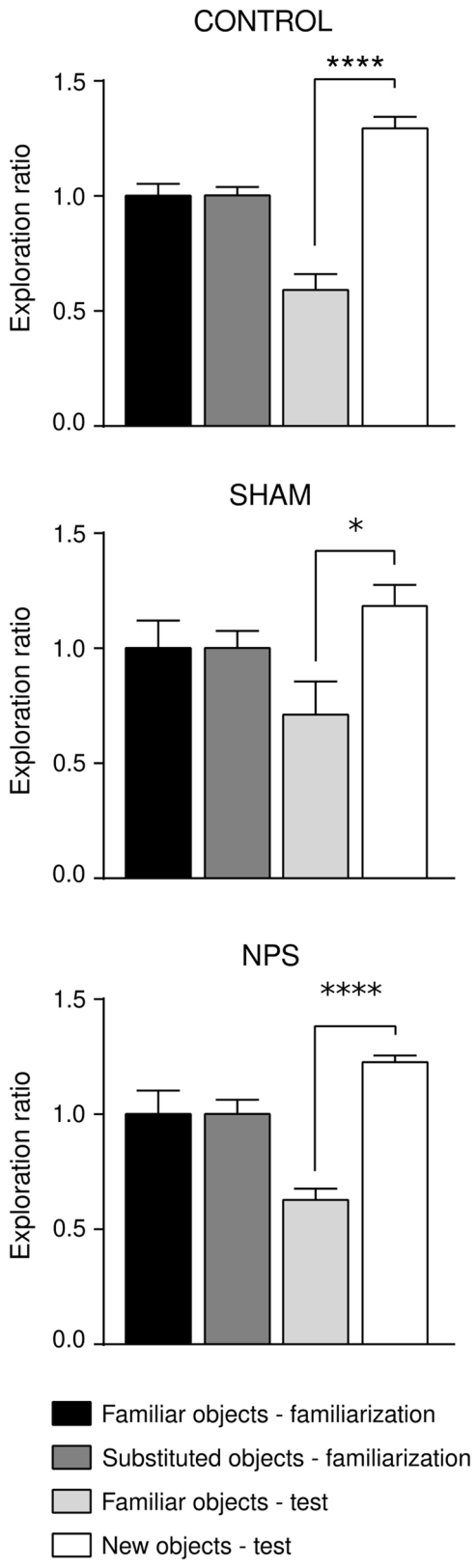

Fig. 2. Results of the object recognition test. Animals of all experimental groups explored new objects during a longer period of time (normalized) when compared to familiar ones (CTRL: 1.29 ± 0.05 (new) versus 0.59 ± 0.07 (familiar), * ** *$p < 0.0001$; SHAM: 1.18 ± 0.09 (new) versus 0.71 ± 0.15 (familiar), *$p < 0.05$; NPS: 1.23 ± 0.03 (new) versus 0.63 ± 0.05 (familiar), * ** *$p < 0.0001$; One-Way ANOVA, post hoc Tukey).

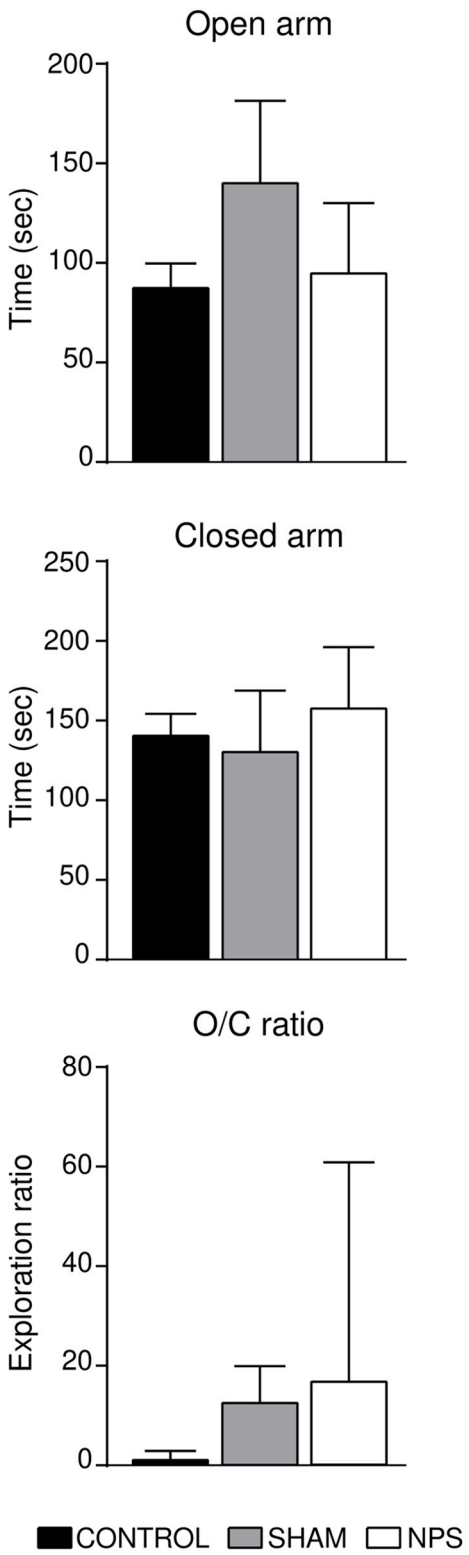

Fig. 3. Results of the elevated plus maze test (EPM). No statistical differences were found among groups regarding time spent exploring open (CTRL: 87 $\pm$ 13 s; SHAM: 140 $\pm$ 42 s; NPS: 95 $\pm$ 36 s; One-Way ANOVA, post hoc Tukey) and closed arms (CTRL: 140 $\pm$ 14 s; SHAM: 130 $\pm$ 39 s; NPS: 157 $\pm$ 39 s; One-Way ANOVA, post hoc Tukey), and also their open to close arms ratio (CTRL: 0.64 (range 0.09 – 2.88); SHAM: 0.45(range 0.0 – 19.9); NPS: 0.32 (range 0.09 – 60.8); Kruskal Wallis, post hoc Dunn). While open and close arm data is depicted as means $\pm$ s.e.m., O/C ratio is shown as boxplots with only 75% percentile and maximum limits being clearly visible.

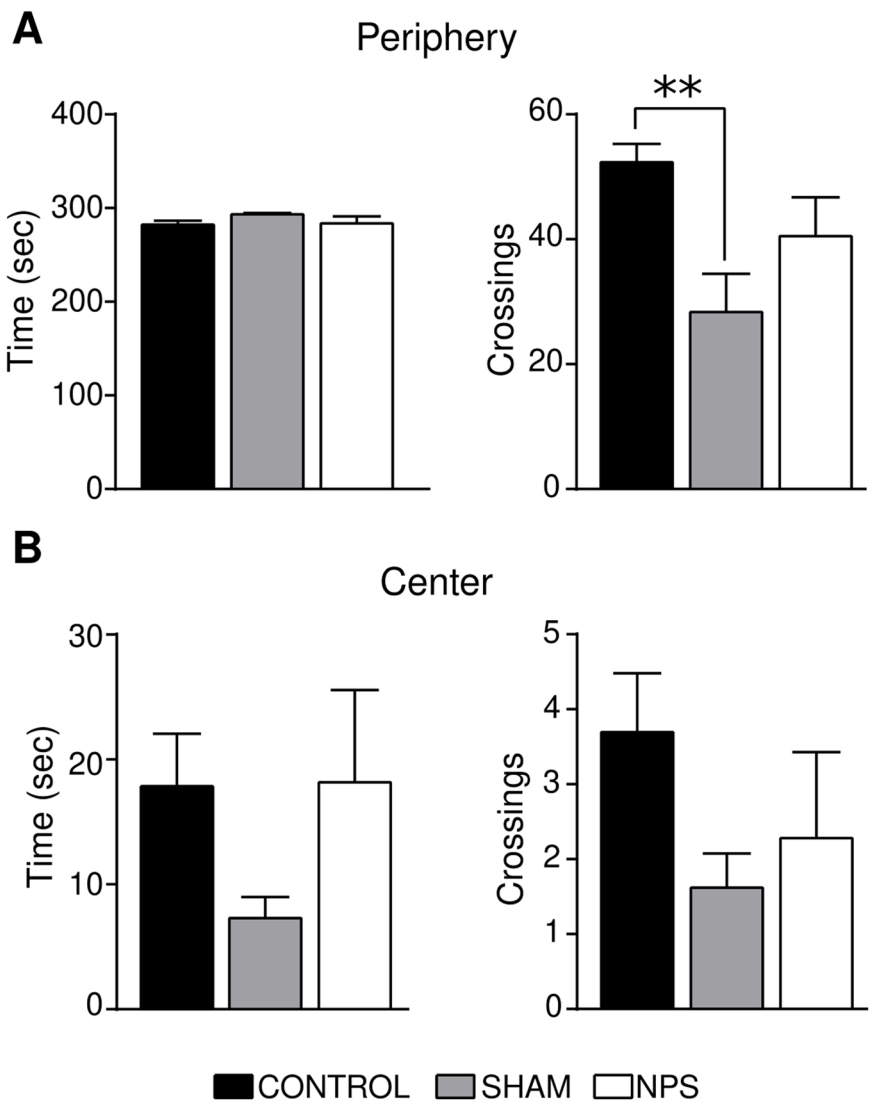

Fig. 4. Results of the elevated open field (OF) test. No statistical differences were found among groups regarding time spent exploring the periphery (CTRL: 282 ± 4 s; SHAM: 293 ± 2 s; NPS: 283 ± 8 s; One-Way ANOVA, post hoc Tukey) or the center (CTRL: 18 ± 4 s; SHAM: 7 ± 2 s; NPS: 18 ± 7 s; One-Way ANOVA, post hoc Tukey) of the arena, nor in the number of crossings in sections of the center (CTRL: 3.7 ± 0.8; SHAM: 1.6 ± 0.5; NPS: 2.3 ± 1.1; One-Way ANOVA, post hoc Tukey). Conversely, sham animals crossed the sectors in the periphery a smaller number of times when compared to control animals (CTRL: 52.3 ± 3.0; SHAM: 28.3 ± 6.2; NPS: 40.4 ± 6.3; **$p < 0.01$, One-Way ANOVA, post hoc Tukey).

of each stage (Fig. 7B right column). Finally, number of transitions between any possible pair of stages were also not significantly different across the eight days of recording (Fig. 7 C and Fig. 7D). Transitions from REM to SWS are less common, while transitions from REM to WK do not occur spontaneously in healthy animals. For this reason, these results were not shown here.

Plots of spectral content in each stage across all days (grouped in pairs for simplicity) are depicted in Fig. 8A (for each stage, top left depicts days 1 and 3, top right 2 and 4, bottom left 5 and 7, and bottom right 6 and 8). Typical LFP spectrum can be observed (Fig. 8A, 8B and 8 C). During WK, most of the content of LFP is distributed across delta and theta bands, with two clear peaks, one in each band, with a theta peak frequency of circa 6.5 Hz. In SWS, there is a clear increase of power in the delta range, particularly in the upper portion of the band (a shallower valley between peaks) with a delta peak frequency of 1.9 Hz. Finally, during REM, there is a clear predominance of theta power with a peak frequency of 7 Hz. EMG power is clearly distinct between stages, WK containing more energy in comparison to SWS and also REM with an even greater difference to this last one (Fig. 8A bottom row). Overall, these features remained unchanged across all eight days of experimentation, including those when NPS was applied (pink shadows in panels of Fig. 8A).

The final set of results follows previous findings. Clusters for each stage were easily identified and detected by the algorithm and were found in the expected positions. In a visual inspection, there were no major deformations of the state map across the days of experimentation, as illustrated by the representative case of Fig. 9 A. The area of the automatically detected clusters did no differ across the days for any stage (Fig. 9B). The general center of mass for the state maps, calculated as the mean X and mean Y of all points, remained very stable along the experiment (Fig. 9C).

4. Discussion

Overall, both behavioral and electrophysiological experiments described in this work have shown no significant differences across groups or days of experimentation with alternating periods of NPS application. Taken together, this is evidence that NPS does not have an impact on neural function or on the global forebrain neurodynamics, at least as far as it has been tested here.

Results from the object exploration test corroborated the innate tendency of healthy animals to explore new objects with greater impetus than familiar ones, as indicated by a statistically preference for No in all groups [40]. It also suggested that neither the surgical procedure nor NPS application caused a deleterious effect on the memory formation process, at least regarding the ability to recognize objects. The EPM test was used to assess the conflict between the natural tendency of rodents to explore new environments and the fear they have related to height

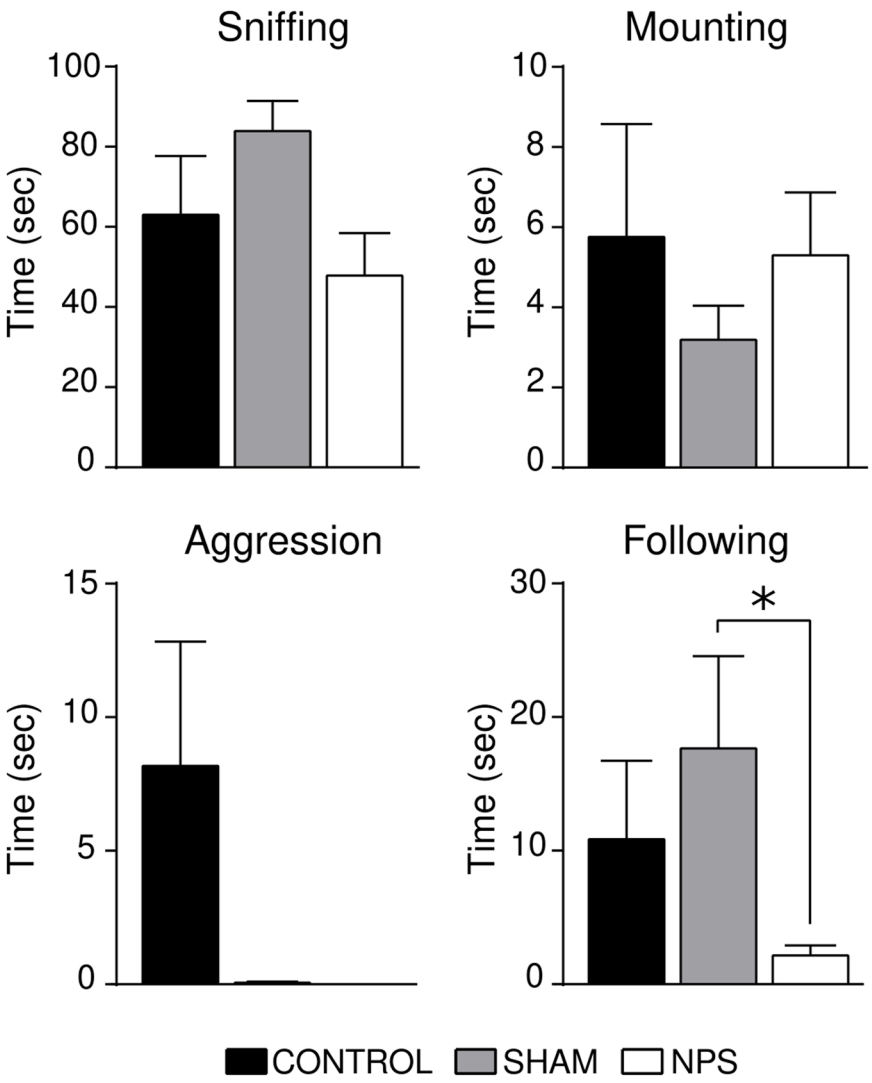

Fig. 5. Results of the social interaction test. No statistical differences were found among groups regarding time spent in sniffing (CTRL: 62 ± 15 s; SHAM: 84 ± 8 s; NPS: 48 ± 11 s; One-Way ANOVA, post hoc Tukey), mounting (CTRL: 5.8 ± 2.8 s; SHAM: 3.2 ± 2.4 s; NPS: 5.3 ± 1.6 s; One-Way ANOVA, post hoc Tukey), and aggression behavior (CTRL: 8.2 ± 4.7 s; SHAM: 0.05 ± 0.05 s; NPS: 0 ± 0 s; One-Way ANOVA, post hoc Tukey). NPS animals spend a smaller amount of time following their pairs when compared to sham animals (CTRL: 10.8 ± 5.9 s; SHAM: 17.6 ± 6.9 s; NPS: 2.1 ± 0.8 s; *$p < 0.05$, One-Way ANOVA, post hoc Tukey).

and open spaces [42]. Less anxious animals tend to explore the open arms of the labyrinth more [43]. Considering time profiles spent by animals from different groups in closed and open arms of the EPM, baselevel anxiety was not affected by the surgical procedure or by NPS. Additionally, OFM results largely corroborated this notion, while also suggesting no significant impact on motor function (although in a very limited way). Finally, SI test data also suggested that neither the surgical procedure nor NPS had an impact on the integrity of the rodents' social behavior.

Electrophysiological and behavioral assessment of the SWC is in line with these observations. There were no detectable alterations in the sleep architecture (periods, proportions, and transitions) and neither the oscillatory content of neural activity nor the global forebrain dynamics changed significantly. On contrary, all parameters measured in this study showed considerable stability across the many days of experimentation. Objective behavioral and electrophysiological characterization of the SWC is an important indicator of preservation of neural function, given that virtually all possible spontaneous patterns of oscillatory activity and behavioral expressions of healthy animals may occur in the period of several daily recording sessions [49]. Thus, observing no changes is a strong indication that NPS does not have an impact on baseline neuronal activity.

These favorable set of results was, in fact expected, once related literature on experimental and clinical brain stimulation frequently reports no impact or even amelioration of neural function. In the study by Creed et al. [51], deep brain stimulation (DBS) was applied to the subthalamic nucleus (STN) and to the entopeduncular nucleus (EPN) of rats. Similar to our results, authors also reported no effects on EPM performance. In individuals with epilepsy, literature on the effects of DBS on neural function is also encouraging. In the study by Salanova et al. [52], DBS was applied to the anterior nucleus of the thalamus (ANT). Some individuals reported impaired memory and mood, but neuropsychological test results indicated improvements in attention, executive function, mood and anxiety disorders, and subjective cognitive function when compared to baseline. Oh et al. [53] also evaluated the cognitive effects of DBS applied to the ANT and found favorable results regarding verbal fluency and verbal memory tasks. According to Inman et al. [54], the direct electrical stimulation of the amygdala increases declarative memory in humans. It is noteworthy that these objective results do not exclude or diminish the relevance of considering individual characteristics and subjectivity since there may be risk factors that predispose the emergence or worsening of disorders and diseases during stimulation [52]. According to Miatton et al. [55], the use of DBS as a treatment for epilepsy increased emotional well-being at the group level, although individual results were too diverse to allow a more viable interpretation. In face of these previous findings, present results are largely plausible and there is no special reason why NPS should be any different, in terms of impacting neural function, to other neuromodulation approaches. On the contrary, as previously discussed, the low-energy feature of the approach and the natural-like temporal pattern could be even more

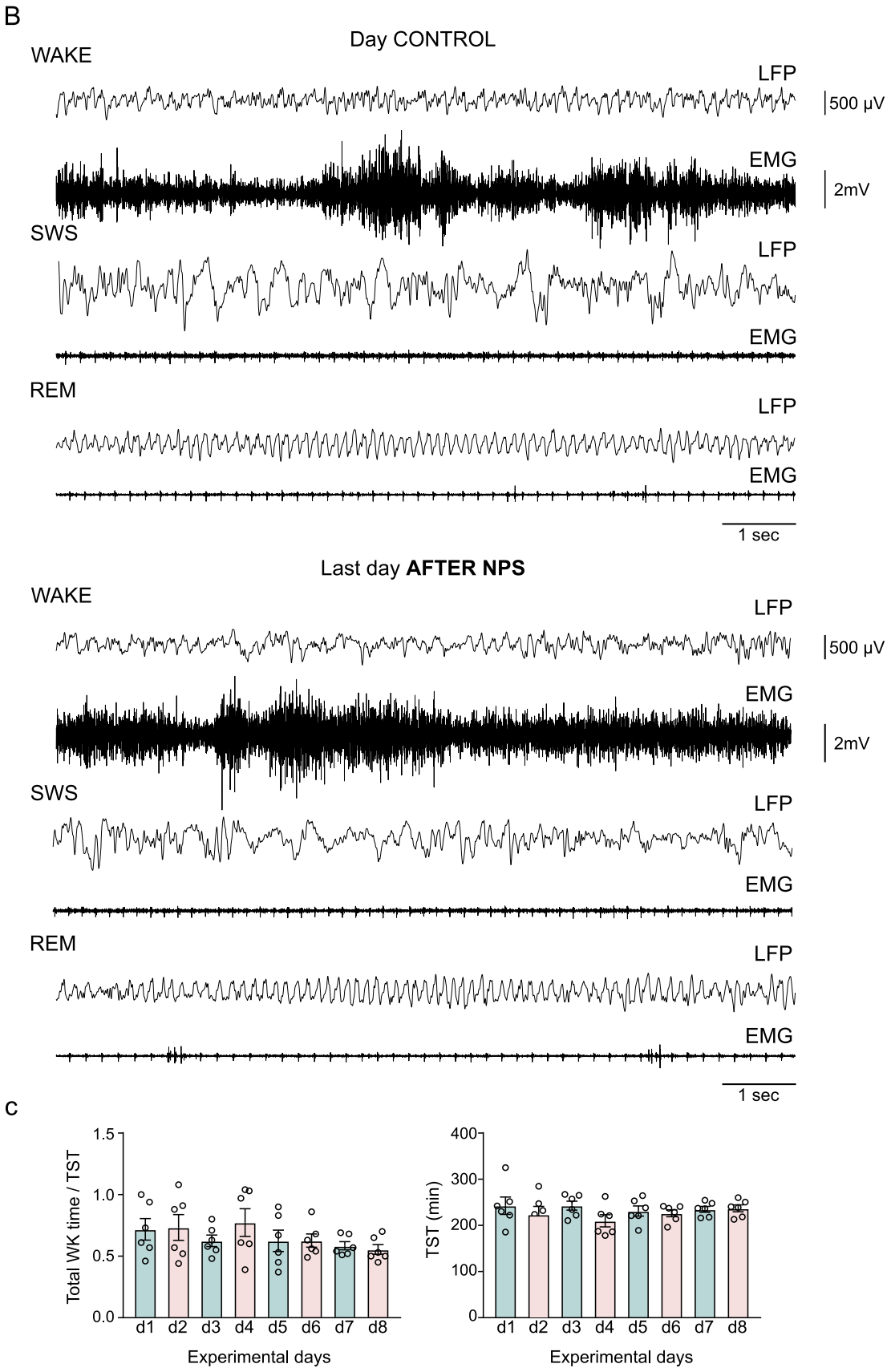

Fig. 6. Electrophysiological results. A) LFP and EMG raw tracings in the first and last days: stages of the sleep-wake cycle (WK, SWS, REM) can be easily identified. B) Proportion of time in wakefulness in comparison to total sleep time (TST; left) and TST (right) along the 8 days of recording.

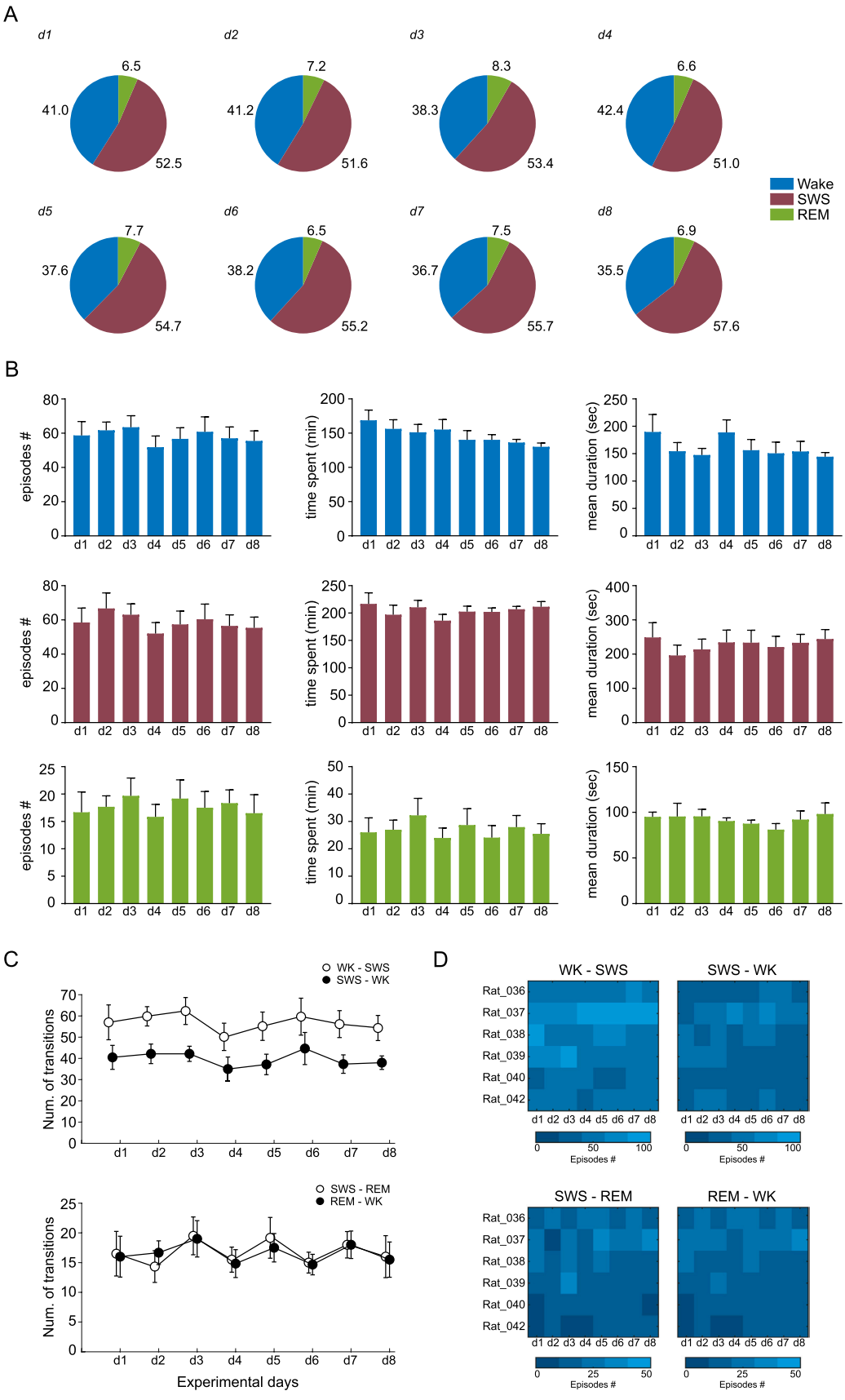

Fig. 7. Sleep-wake cycle architecture. A) Proportion of time spent in each stage along the 8 days of electrophysiological recording. B) Number of episodes (left column), total time spent (middle column), and mean duration of episodes of each sleep-wake cycle stage, wakefulness (WK: top row), slow-wave sleep (SWS: middle row), and rapid eye movement (REM: bottom row) sleep. C) Number of transitions from WK to SWS (white circles) and back (black circles) and from SWS to REM (white circles) and back (black circles are depicted in top and bottom panels, respectively. Uncommon or unseen transitions between WK and REM are omitted. D) Transitions between stages are shown for each animal as pseudo-color graphs.

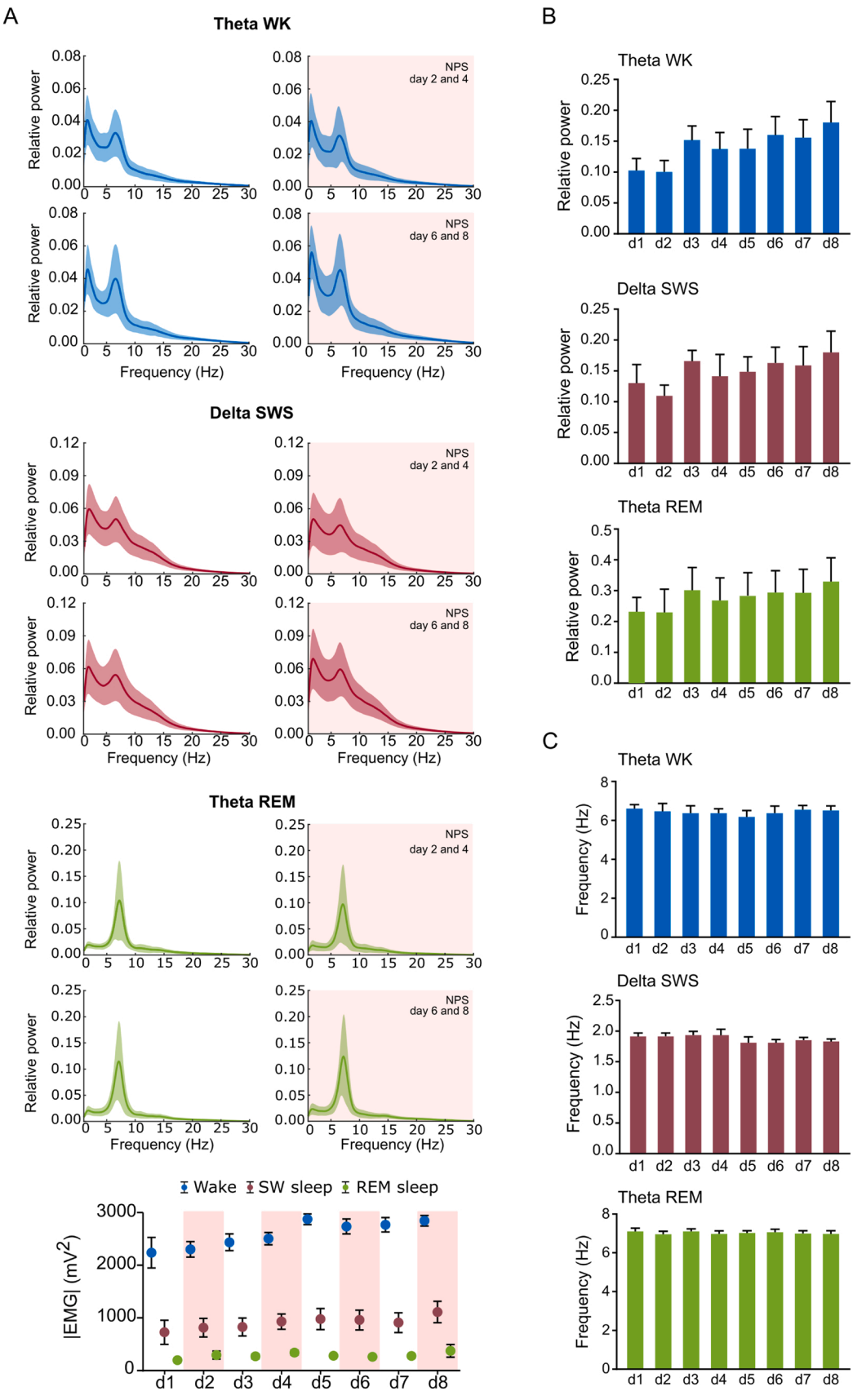

Fig. 8. Spectral analysis of electrophysiological recordings during the sleep-wake cycle. A) Spectra of animals during WK, SWS, and REM are organized in four panels for each stage: top left, days 1 and 3; top right, days 2 and 4; bottom left, days 5 and 7; bottom right, days 6 and 8. Bottom panel shows total power of EMG recording in the different sleep-wake cycle stages along the 8 days of experiment. Pink-shadowed plots represent days in which animals underwent application of NPS. B) Relative spectral power during sleep-wake cycle stages in bands of interest: WK and REM, theta (5 – 9 Hz); SWS, delta (0.5 – 4 Hz). C) Values of peak frequency in the bands of interest remain the same across the experiment.

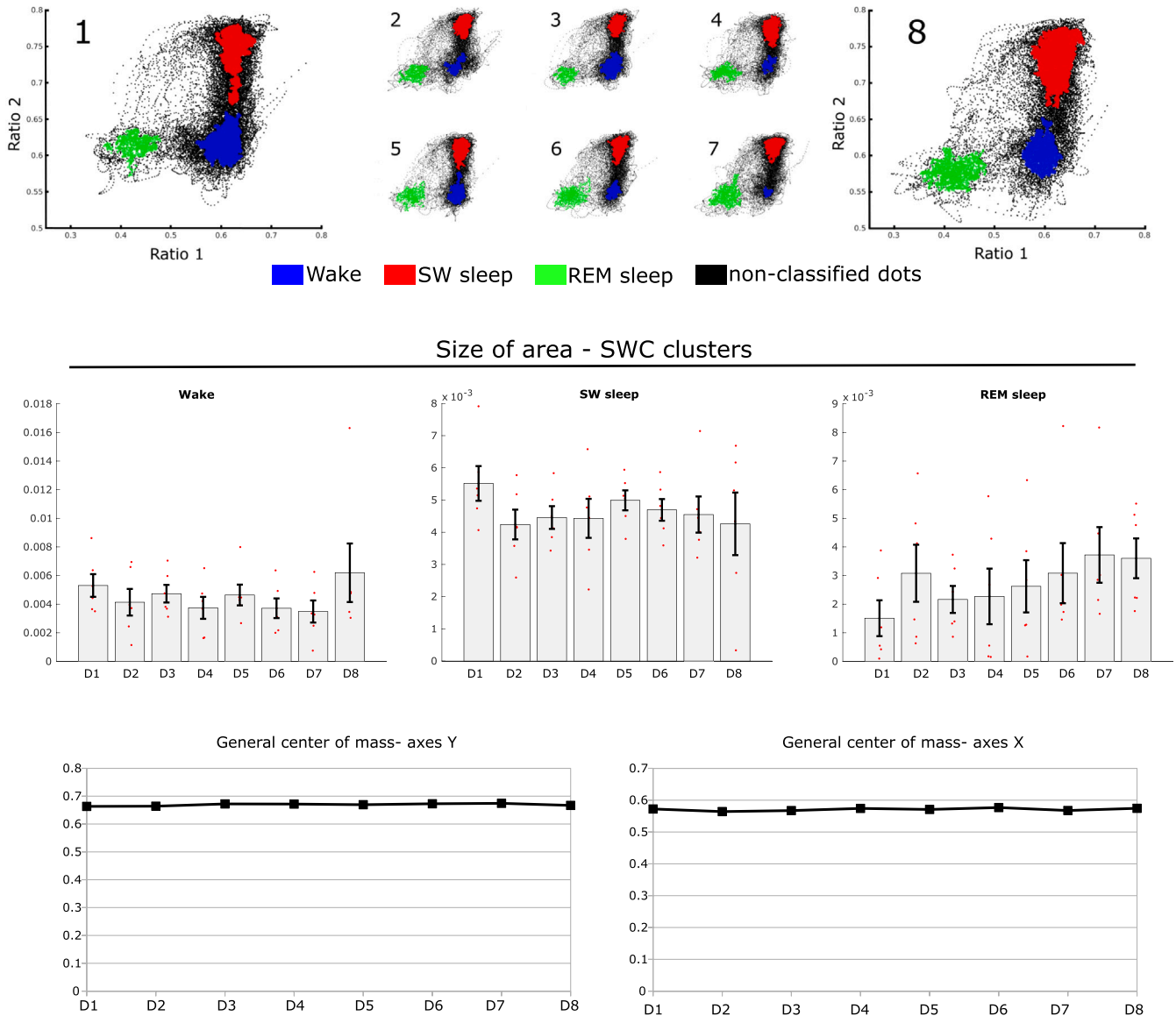

Fig. 9. Global forebrain neurodynamics as depicted by state maps of spectral content. Top panels depict state maps for all the 8 days of experiment. Panels in the middle row show the area of clusters of points for each of the sleep-wake cycle stage (WK, left; SWS, center; REM, right). Bottom panels show the general center of mass coordinates (x, abscissa at left; y, ordinate at right).

beneficial in this aspect of overall safety. Although DBS is a valuable treatment alternative for patients with refractory epilepsy and does not appear to have major neuropsychological consequences, neurosurgical interventions involve risks such as hemorrhage, epileptic seizures, pneumonia, and infections [56]. Considering there would be no major change in the surgical procedure to deliver NPS, it follows that this approach bears, at most, the same level of such risks.

There have been a few exceptions to the "no effect" behavior among present results and some statistically significant differences were found. Sham animals displayed a smaller number of crossings in the periphery of the OFM when compared to controls. Additionally, sham animals spent a larger amount of time in the "following" behavior during SI test in comparison to NPS treated animals. These finding are of unclear significance and, in our understanding, possibly irrelevant, for several reasons. First, they do not point towards any clear effects in terms of the studied neural functions. Moreover, they are not related to the treated group but mostly to sham animals. Particularly, a statistically significant enhance of the "following" behavior in sham animals appeared only when compared to a decreased group value for NPS animals, while both were not significantly different from controls. It is thus plausible that these differences might be mere statistical fluctuations considering the great number of distinct tests and experiments performed here. In any case, further investigation is certainly needed in order to better elucidate this issue.

The understanding that NPS has no significant impact on neural function is, of course, constrained by the number and limitations of the tests carried out here. A much broader scope of behavioral assessment would be ideal. Yet, this is not only hardly feasible but also faces important ethical implications given the number of animals needed to explore many possibilities. In our understanding, the set of tests employed in this study encompasses neural functions of major importance supported by major and distinct neural circuits in the brain. Together with the electrophysiological investigation carried out in our experiments, present results are sufficient to provide first robust evidence that NPS does not significantly affect brain physiology. On the other hand, investigation of other functions such as a thorough motor and cognitive testing (e.g., You et al. [57]) and assessment of neurobiological markers for neural activity (c-Fos), plasticity (immediate early

genes and neurotrophic factors), neurogenesis, and even neuronal death may yield novel and important results to fully support a safe translation of the method to humans in future clinical trials.

It is well known that the amygdala is a structure more directly related to aversive memory [34], and injuries in this region can hamper the acquisition of this specific type of memory trace [58], rendering such evaluation equally important. Aversive memory can be promptly studied in animals using well stablished protocols such as fear conditioning or two-way active avoidance task. Our group is, in fact, carrying out such experiments to evaluate the impact of NPS in fear processing within the scope of a broader investigation that also assesses putative beneficial effects on pathological anxiety, the results of which have been preliminarily published elsewhere [59].

Overall, present results indirectly corroborated the notion that NPS acts by suppressing aberrant levels of brain synchronization in an on-demand-only fashion, as initially postulated. If it had an unspecific desynchronizing effect, this would be seen in the LFP spectral content or in the state maps of global forebrain neurodynamics. Also, considering the importance of intricate coordination of synchronous rhythms for sleep generation, memory formation and many other neural functions [60,61] disturbances in synchronization levels would not only implicate in changes of electrophysiological parameters investigated, but also in the expression of spontaneous behavior, which was not the case.

Although the experimental design of this work aimed at proving that an effective strategy against epilepsy, using non-periodic intracephalic microstimulation, would not compromise key physiological functions due to treatment side-effects, perhaps a broader interpretation of our findings might be useful to other neurological conditions as well. In addition to what has been described before, the amygdala also interacts with prefrontal regions in order to interface emotions with higher cognitive processes. While its increased activation would most likely correlate with behaviors such as aggressiveness, fear, sex, and feeding, recruitment of the ventromedial prefrontal cortex (vmPFC) and the dorsolateral prefrontal cortex (dlPFC) would act as bidirectional modulators of such functions. Conversely, Mourão and colleagues have shown that aspects related to temporal coding are as important as stimulation frequency and intensity for directing amygdala output to functional competing structures such as hypothalamus or prefrontal cortex [62]. Thus, the persistence of neural function seen in our present results suggests that no particular amygdalar output pathway is being favored by NPS, say, for example, the hypothalamic (dorso-medial hypothalamus) – periaqueductal gray area – brainstem sympathoexcitatory pathway. In fact, due to its pseudo-random temporal coding, NPS could be working as a non-specific pattern generator that could potentiate the midcingulo-insular network (i.e. salience network), switching brain activity from default mode network to central executive network state. The resulting consequence would be potentiated self-awareness, social behavior, communication, emotional/cognitive processing, and overall sensory-motor integration. Thus, considering the known overlap between broad circuitries executing related functions, as mirror-like observational motor function [63], such on-demand-only interference can be a desirable approach to pathologies treated by electrical stimulation. In any case, understanding the mechanisms by which stimulation temporal patterns used in this and other studies interfere with neural network recruitment and modulation could contribute to new insights into the exploration of human neurophysiology, pathophysiology, and even rehabilitation neurotechnology [64–66].

5. Conclusion

In this study, we investigated the impact of the application of bilateral asynchronous NPS to the basolateral amygdala in major neural function and global forebrain neurodynamics. Data have shown no significant modification in behavioral and electrophysiological parameters measured, suggesting that neural function is preserved in animals submitted to NPS treatment. Given the considerably broad scope of tasks and analyses carried out, which encompass diverse neural functions, distinct neural circuitry, and many different brain states, results are an indication of the safety of the protocol with important implications to its translational potential. Finally, although they do not represent additional proof, present findings can be easily accommodated inside the understanding that NPS works like an on-demand-only desynchronizing stimulus for its therapeutic effect. Additional investigation, particularly regarding effects on aversive memory and pathological anxiety, which are highly dependent on the function of amygdala, is certainly needed for a more comprehensive understanding of the effects of NPS.

CRediT authorship contribution statement

Larissa Altoé Réboli: Conceptualization, Methodology, Formal analysis, investigation, Data curation, writing – original draft. **Renato Marciano Maciel**: Conceptualization, Methodology, Formal analysis, Investigation. **Jasiara Carla de Oliveira**: Investigation. **Márcio Flávio Dutra Moraes**: Conceptualization, Resources, Writing – review & editing. **Cristiane Queixa Tilelli**: Methodology, Resources, Writing – review & editing. **Vinícius Rosa Cota**: Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest

None.

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Author contributions

L.A.R, R.M.M., C.Q.T., M.F.D.M., and V.R.C. conceived the presented idea. **L.A.R., R.M.M., and J.C.O.** performed the experiments. **L.A.R and V.R.C** wrote the manuscript. **C.Q.T. and M.F.D.M** provided critical feedback and helped shape the manuscript. **V.R.C.** acquired the funding and supervised the project. All authors reviewed, edited and approved the paper.

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