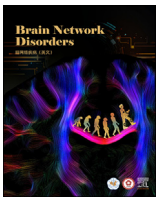
Brain Network Disorders xxx (xxxx) xxx

Contents lists available at ScienceDirect

Brain Network Disorders

journal homepage: www.sciencedirect.com/journal/brain-network-disorders



Original Article

Synchronization-suppressing stimulation of the amygdala circuitry reduces pathological anxiety in sub-chronic stress rat model

Larissa Altoé Réboli [a], Vinícius Rosa Cota [a,b,*]

[a] Laboratory of Neuroengineering and Neuroscience (LINNce), Department of Electrical Engineering, Federal University of São João Del-Rei, São João Del-Rei, MG, 36307-352, Brazil

[b] Rehab Technologies Lab, Istituto Italiano di Tecnologia, Liguria, Genova, 16163, Italy

ARTICLE INFO

Keywords:
Temporally complex stimulation
Anxiety
Neural network
Neural synchronization
Electroceuticals

ABSTRACT

Background: Synchronization across neural circuits is inextricably associated with brain function and pathology. Although not largely explored, this framework can be applied to baseline anxiety and its disorder, which is characterized by aberrant levels of synchronization between the amygdala nuclei and other areas of the extended amygdala, particularly the bed nucleus of the stria terminalis (BNST) and those outside this complex. Here, we aimed to test the hypothesis that a temporally complex form of electrical stimulation (non-periodic stimulation [NPS]) of the amygdala, specifically designed to disrupt hypersynchronous activity in epilepsy, a major comorbidity of pathological anxiety, may reduce its symptoms.

Methods: Wistar rats were subjected to a physical restriction protocol model of stress to induce pathological anxiety and were assessed using the gold standard elevated plus maze (EPM) and open field (OF) tests.

Result: In all criteria measured by the tests, NPS animals displayed reduced levels of anxiety-related symptoms, back at physiological levels.

Conclusions: Considering the known effects and mechanisms of NPS on epileptic phenomena, we hypothesized that the therapeutic effects were achieved by desynchronization (or normalization of synchronism levels) across brain circuits involving the amygdala, BNST, and others. Overall, past and present findings suggest that NPS may be considered as a therapeutic alternative for the treatment of anxiety disorders.

1. Introduction

It is now well accepted that the dynamic entrainment of brain structures into functionally coherent neural circuits underlies brain function. In fact, a series of different neurobiological mechanisms, ranging from molecular to cellular, and even neural network levels, responsible for the induction of uni- or bi-directional driving forces, a process generally known as neural synchronization, have been identified and characterized.[1,2] Although with different terminologies, such universal mechanisms have been described in many scenarios, including sleep,[3,4] memory consolidation,[5,6] cognition,[7,8] and somatosensory processing.[9] Moreover, neurological disorders are often described as dysfunctions, exaggeration, or deficits of neural synchronization and,[10] simultaneously, treatments, pharmacologically or not, specifically tailored to modulate them have been put forward.[11]

This rationale is particularly important for designing therapeutic neurostimulation strategies. For instance, coordinate reset stimulation was designed to deliver pulsatile stimuli in specific phases of background synchronizing neural oscillations to induce resetting and thus desynchronization.[12,13] Similarly, the induction of synchronization of oscillations across hemispheres by non-invasive brain stimulation has been used to improve cognitive performance in patients with memory impairment.[14] In fact, fine-tuning synchronism levels within neural circuits is one of the most important aspects of novel neurostimulation techniques that incorporate disruptive technologies such as neuromorphic technology and closed-loop personalization.[15,16]

In line with this reasoning, non-periodic stimulation (NPS) has been proposed as a means of suppressing the aberrant synchronization observed in epileptic phenomena, thus providing a therapeutic alternative to refractory epilepsy. The rationale is that a temporally complex pattern of stimulation impairs the aberrant coupling of brain structures to hypersynchronous epileptiform activity.[17] NPS applied to the basolateral amygdala (BLA) has been shown to suppress acute and chronic spontaneous seizures in animal models,[18,19] while displaying no overt effects on

* Corresponding author. Rehab Technologies Lab, Istituto Italiano di Tecnologia, Via Morego 30, Liguria, Genova, 16163, Italy.
E-mail addresses: vrcota@ufsj.edu.br, vinicius.rosacota@iit.it (V.R. Cota).

https://doi.org/10.1016/j.bnd.2024.07.001
Received 19 April 2024; Received in revised form 1 July 2024; Accepted 8 July 2024

Please cite this article as: Réboli LA, Cota VR, Synchronization-suppressing stimulation of the amygdala circuitry reduces pathological anxiety in sub-chronic stress rat model, Brain Network Disorders, https://doi.org/10.1016/j.bnd.2024.07.001

neural function mediated by the target substrate.[20] Mechanistic studies have suggested a synergy between direct and indirect network-level effects.[21–27] This seems to be potentiated by the scale-free natural-like aspect of the stimulus, which putatively induces normalization of synchronization levels instead of straightforward desynchronization.[28] Such action may be highly beneficial as it is evoked in an "on-demand" only fashion,[29] preserving basal functioning of the circuitry, which is compatible with behavioral and electrographic observations.[20]

The brain substrate chosen as the target for NPS in these studies, the BLA, certainly played a major role in the observed effects.[22,30] The amygdala, with its intricate cytoarchitectonics and widespread network connectivity, is a determinant of epileptic phenomena and its treatment.[31–37] Notably, the amygdala is involved in a series of important neural functions, including processing of emotions in general and anxiety in particular.[38,39] There is mounting evidence that the intricate neural circuitry composed of its nuclei, including areas of the extended amygdala such as the bed nucleus of the stria terminalis (BNST), in connection with several other brain substrates, plays fundamental and complementary roles in the control of fear, anxiety, and behavioral expressions.[40] Moreover, while synchronization processes of such networks mediate these functions at healthy levels,[41,42] aberrations can lead to dysfunction.[43] This can be expressed as neurological disorders such as stress, pathological anxiety, and panic. In all of these cases, amygdala dysfunction was observed,[44–46] which was mostly characterized by hyperexcitability and hypersynchronism.[47]

Considering the importance of the amygdala circuitry, including its afferents and efferents, in both physiological and pathological anxiety, and the observed effects of scale-free stimulation in the normalization of synchronism levels, it is plausible that the application of NPS to the amygdala may have a beneficial impact on the signal and symptoms observed in an animal model of the disorder. We tested this hypothesis in rats by subjecting them to sub-chronic stress induced by physical restriction. Anxiety levels were measured using gold standard behavioral tasks, such as the open field (OF) test and elevated plus maze (EPM), during acute NPS stimulation. The results suggested that NPS had a therapeutic effect against pathological anxiety, putatively mediated by circuitry desynchronization.

2. Methods

2.1. Animals and groups

In this study, 39 male Wistar rats weighing 250 and 380 g were used. The animals were sourced from the central animal facility of the Federal University of São João Del-Rei (UFSJ) and kept in a laboratory vivarium during the experiment, with a 12-hour light–dark cycle (lights on at 7 a.m. and off at 7 p.m.). The animals were provided with *ad libitum* food and water and were housed at an average temperature of 23°C ± 2°C. They were randomly distributed into the following four experimental groups: (1) control (CTRL, $n = 14$): animals without any intervention; (2) stressed control (S-CTRL, $n = 10$): animals subjected to the specific treatment according to the stress protocol (SP); (3) stressed surgical control (S-SHAM, $n = 7$): animals subjected to (a) the implantation procedure of electrical stimulation electrodes without stimulation and (b) the specific treatment according to the SP; and (4) stressed and stimulated with NPS (S-NPS, $n = 8$): animals subjected to (a) the specific treatment according to the SP and (b) non-periodic electrical stimulation pattern (thus also to surgical implantation of electrodes) [Fig. 1]. While the CTRL group was used to assess baseline levels of anxiety in the animals, S-CTRL enabled the measurement of pathological levels to validate the stress model. Furthermore, while S-NPS *vs.* non-stimulated groups tested the effects of the stimulation, S-SHAM was performed for the control of secondary effects that could be caused by the implantation of electrodes into the amygdala.

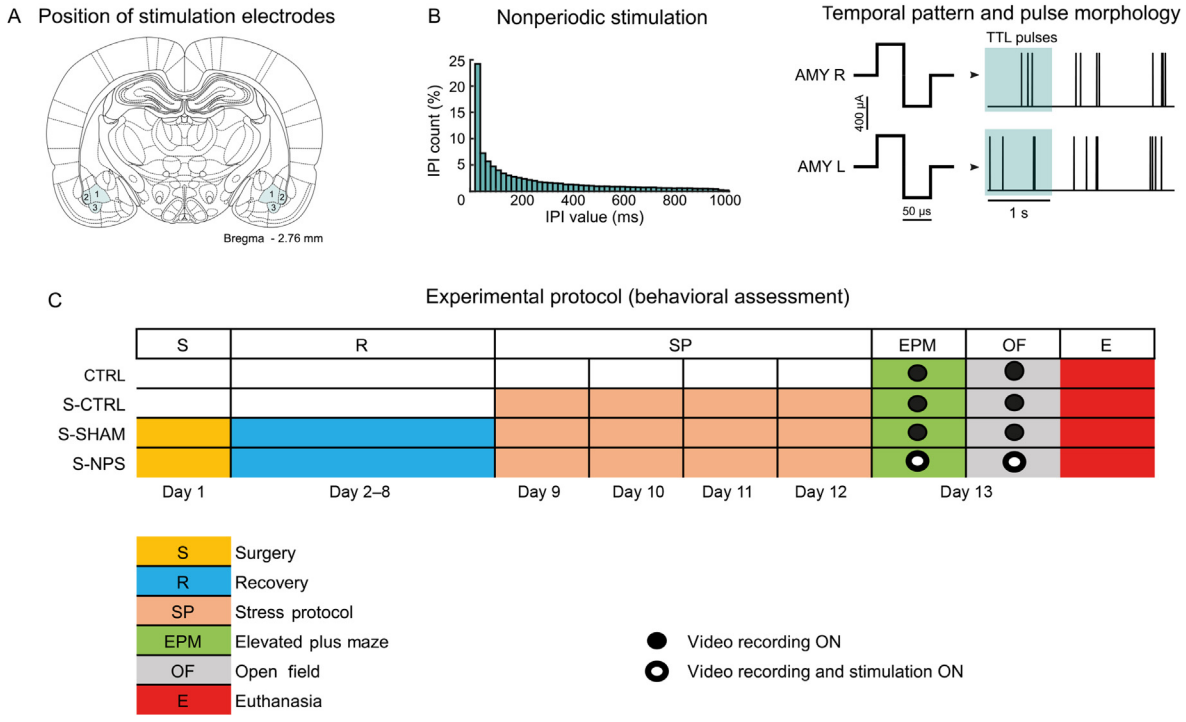


Fig. 1. Details of the experimental protocols. (A) The bilateral amygdala as a substrate for NPS application. (B) NPS temporal pattern with its IPI distribution (left), presentation of TTL control pulses across time, pulse morphology, and asynchronous application to the right and left amygdala (right). Colored areas refer to basolateral amygdaloid nucleus anterior part (BLA, area 1), posterior part (BLP, area 2), and ventral part (BLV, area 3). (C) Chronology of behavioral tests as applied to each animal group. AMY L: Left amygdala; AMY R: Right amygdala; CTRL: Control; E: Euthanasia; EPM: Elevated plus maze; IPI: Interpulse interval; NPS: Non-periodic stimulation; OF: Open field; R: Recovery; S: Surgery; S-CTRL: Stressed control; S-NPS: Stressed and stimulated with non-periodic stimulation; SP: Stress protocol; S-SHAM: Stressed surgical control; TTL: Transistor-transistor logic.

The sequence of the procedures for each experimental group is shown in Fig. 1C. Surgery for the implantation of electrodes (S-SHAM and S-NPS groups) was performed on day 1 of the protocol, followed by a 7-day recovery period (days 2–8; *cf*. Section Surgical procedure for the implantation of electrodes). Subsequently, the SP was performed (groups S-CTRL, S-SHAM, and S-NPS) for the following 4 days (days 9–12; *cf*. Section Physical restriction-induced stress protocol). Behavioral tests (all groups; *cf*. Section Behavioral evaluation of anxiety) were performed on day 13. Electrical stimulation was applied concomitantly with behavioral testing (S-NPS group; *cf*. Section Electrical stimulation of the basolateral amygdala). Finally, the animals were euthanized, and their brains were removed for histological processing (*cf*. Section Histological procedures). The SP and behavioral tests were conducted between 8 a.m. and 1 p.m.

All procedures were approved by the Ethics Committee on Animal Use (CEUA) of the UFSJ under protocol (Nos. 025/2015 and 023/2016), and were in accordance with international guidelines for the care of animals in research. Animals were always male to avoid known hormonal factors that vary across the estrous cycle in females, decreasing data variability and reducing the total number of animals.

2.2. Surgical procedure for the implantation of electrodes

For electrical stimulation of the BLA, the S-NPS group underwent bilateral implantation and fixation of electrodes in the BLA. S-SHAM animals also underwent electrode implantation as a control for the effects of the surgery. The procedure began with the induction of anesthesia with ketamine (100 mg/kg; Konig do Brazil, Santana do Paraíba, Sao Paolo, Brazil), xylazine (5 mg/kg; Syntec do Brazil, Cotia, Sao Paolo, Brazil), and fentanyl (0.025 mg/kg; Union Chemical do Brazil, Londrina, Paraná, Brazil). After trichotomy and appropriate aseptic measurements, the animals were placed in a stereotaxic apparatus. Next, the skin of the head was cut open, and after all the subcutaneous material was removed, a craniotomy was performed. Bipolar electrodes (set up in twisted pairs, with a distance of 0.5 mm between the tips), made of stainless-steel wires coated with Teflon (diameter 127 μm, model #791400, A-M Systems Inc., Sequim, Washington, USA) were then implanted in both the right and left amygdalae. The electrode positions were determined according to the coordinates provided by the neuroanatomical atlas of Paxinos and Watson[48] (antero-posterior = −2.8 mm, medio-lateral = ±5 mm relative to Bregma, and dorso-ventral = 7.2 mm from the dura mater) and were reached with the aid of the stereotaxic apparatus coordinate axes (Insight Equipamentos Ltd., Ribeirão Preto, Sao Paolo, Brazil) [Fig. 1A]. The electrodes were anchored to the skull using zinc cement (Vigodent-Coltene Company, Rio de Janeiro, Rio de Janeiro, Brazil) and soldered to a 6-pin RJ-11 type connector. This connector was fixed to the animal's skull using self-polymerizing acrylic (Artigos Odontológicos Clássico Ltd., Sao Paolo, Brazil). Seven days after postoperative recovery (days 2–8), the animals underwent the experiments.

2.3. Electrical stimulation of the basolateral amygdala

For NPS of the BLA, a fixed current electrical stimulator composed of a control unit (model #3800, A-M Systems Inc.) and two isolation units (model #3820, A-M Systems Inc.) was used. The stimulation waveform consisted of biphasic square pulses (phase-balanced, zero net charge), four pulses every second, and pseudorandomized in a power-law distribution [Fig. 1B, left panel], with a total duration of 100 μs and variable amplitude between 100 μA and 600 μA per phase according to the animal's susceptibility [Fig. 1B, right panel]. A susceptibility to stimulus test was performed prior to stimulation, and the current value was set as the maximum amplitude that did not evoke overt effects (or very minimal effects) on general behavior when very low-frequency (<0.1 Hz) isolated pulses were applied. NPS was delivered bilaterally to the BLA, asynchronously between the hemispheres [Fig. 1B, right panel]. Stimulation was applied only to the S-NPS group for the total duration of the behavioral tests [Fig. 1C], amounting to 10 min or 2400 pulses. A more detailed description of NPS can be found in Cota *et al.*[18]

The stimulation target of this study was the same as that chosen for our previous investigations of the effects of NPS in suppressing seizures: the amygdala or, more specifically, the basolateral nucleus. The rationale for this choice was related to both its anatomy and function. The BLA is characterized by extensive interconnections with higher-order cortical areas in the prefrontal, temporal, insular, and hippocampal cortices. It is the main target of sensory inputs. It then projects to the central nucleus, BNST, and ventral striatum, which in turn activate the hypothalamus, brainstem, and other regions to generate somatomotor, autonomic, and endocrine components of emotional and motivational behavior. Circuits involving the BLA are critical for aversive behaviors, including fear conditioning, fear extinction, and anxiety.[49] It is also essential for the expression of epileptic phenomena and, as mentioned, it plays a central role in the therapeutic effect of NPS.[30]

2.4. Physical restriction-induced stress protocol

To induce pathological anxiety, the animals were immobilized in a transparent plastic pastry bag, similar to Decapicones, measuring 37 × 18 cm, with a frontal cutout for breathing. Immobilization was performed for 1 hour per day for 4 consecutive days. During this 1 hour, the animals were deprived of water and food.[50–52] The sub-chronic physical restriction model offers several advantages, including high reproducibility and precise control over the stressor, ensuring consistent application across studies. It is ethically favorable because of the shorter duration of stress exposure and is less resource-intensive than other chronic stress models. This model effectively induces both behavioral and physiological changes relevant to anxiety, mimicking aspects of human chronic stress, while maintaining simplicity and cost-effectiveness. Behavioral tests were conducted only after the stress-inducing protocol was completed on day 13. Data from the CTRL and S-CTRL groups were assessed separately to validate the SP, given its moderate intensity.

2.5. Behavioral evaluation of anxiety

To assess anxiety levels, EPM and OF behavioral tests were conducted. The S-NPS group underwent electrical stimulation during the behavioral tests. The procedures performed in each group and their sequences were shown in Fig. 1C.

In the EPM test, the animal's stay in the maze arms lasted for a single 5-min session, according to standard protocols.[53,54] The time spent in the open arm (OA) and closed arm (CA) was assessed. Data were tabulated semi-automatically (manual entry with automated summation) using PlusMz freely shareable (upon request to the authors) software developed by a collaborator. In the OF test, conducted in a standard circular arena, the duration of the animal's stay in the periphery and center of the arena was measured during a single 5-min session. Similarly, this test evaluates locomotor activity based on the number of crossings between quadrants.[53,55] Data were computed semi-automatically (manual entry with automated summation) with the assistance of OpenFLD freely shareable (upon request to the authors) software, also developed by the same collaborator.

After each experiment, the apparatus were sanitized for subsequent animal tests. Behavioral evaluations were video recorded and later reviewed for data computation and tabulation for statistical analyses.

2.6. Histological procedures

At the end of the experiments, all the animals were euthanized using an overdose of isoflurane gas or intraperitoneal injection of ketamine and xylazine. The brains of animals from the groups that received electrode implants were removed and preserved in 10% formaldehyde for subsequent histological processing. Brain sections were prepared using a freezing microtome. The coronal sections were 50 μm thick, mounted on

gelatinized slides, and stained with cresyl violet (Nissl stain, not shown). To verify the position of the electrodes, the sections were examined using a stereoscopic magnifying glass. Only animals with correctly positioned electrodes were used in this study.

2.7. Statistical analysis

Parametric data were analyzed using one-way analysis of variance (ANOVA), 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 conducted using Dunn's *post hoc* test. For simple comparisons, Student's *t*-test and Mann–Whitney *U* test were used for parametric and non-parametric data, respectively. The results are presented as mean ± standard error of the mean (SEM) or median (range) for parametric and non-parametric data, respectively, and were considered statistically significant when $P < 0.05$.

3. Results

3.1. Anxiety levels increased in stressed models

The first part of the data analysis involved comparison of the data from the CTRL and S-CTRL groups to validate the SP. Significant differences were identified between the groups in both behavioral tests. In the EPM test [Fig. 2], the S-CTRL group spent significantly less time in the OA [Fig. 2A] and more time in the CA [Fig. 2B] ($P < 0.001$ and $P < 0.0001$, respectively). The ratio of the time spent in the OA and CA (OA/CA ratio) was also significantly lower in the S-CTRL group ($P < 0.001$) than in the CTRL group [Fig. 2C].

In the OF test [Fig. 3], there were no significant differences between the groups in the time spent in the periphery [Fig. 3A] or center [Fig. 3B]. However, differences were identified in the number of crossings, both in the periphery ($P < 0.01$) [Fig. 3C] and center ($P < 0.05$) [Fig. 3D]; the S-CTRL group exhibited a lower number of crossings in both cases.

3.2. NPS reduced signals of pathological anxiety back to control levels

In this sequence, we assessed results related to the effects of NPS on anxiety. In the EPM test [Fig. 4], all comparisons showed significant differences between the groups. In the OA, the S-NPS group remained longer than the S-CTRL group ($P < 0.05$) [Fig. 4A], whereas the opposite was observed in the CA ($P < 0.01$) [Fig. 4B]. The ratio of time spent in each arm was significantly higher in the S-NPS group than that in the S-CTRL group ($P < 0.05$) [Fig. 4C]. In all three measurements, there were no statistically significant differences in pairwise comparisons involving the S-SHAM group, except with the CTRL in the CA ($P < 0.05$). There were no statistically significant differences in comparison with the S-SHAM group, except with the CTRL in the CA. Furthermore, the activity levels of the stimulated group were comparable to those of control animals.

In the OF test, significant differences between the groups were found in the time spent in the periphery and center of the arena [Fig. 5]. The S-NPS group explored the periphery for a shorter time and the center for a longer time than the S-CTRL group ($P < 0.05$) [Fig. 5A and B]. There was no significant difference between the groups in the number of crossings in the periphery or center, although there seemed to be a strong tendency in this last measurement [Fig. 5C and D]. Furthermore, there were no differences in the total number of crossings across the zones [Fig. 5E and F].

4. Discussion

In this study, we tested the hypothesis that a scale-free temporally complex pattern of electrical stimulation (NPS), specifically designed to desynchronize the neural circuits applied to the BLA, can reduce the symptoms of pathological anxiety. Therefore, a standard model of stress induced by sub-chronic physical restrictions was employed. Other versions of stress-induction protocols exist, in which animals are restricted for far longer times and across more days. However, for ethical reasons, we reduced the session time and number of days to a viable minimum. Therefore, initial validation of the model standardization was of paramount importance. As shown in Figs. 2 and 3, the experimental design was sufficiently intense to induce stress and pathological anxiety. In fact, it has been broadly reported that animals naturally tend to stay longer in the CA of the EPM or in the outer area of the OF, a behavior that is exacerbated by increased levels of fear and anxiety.[53] Another consequence of such a dysfunctional state is the decrease in locomotion in both the periphery and center of the OF, which was also observed.

After validating the experimental model of pathological anxiety, we investigated whether NPS could reverse stress symptoms displayed by animals. The results strongly suggested that this strategy was able to reduce the symptoms of aberrant anxiety, leveling them back to baseline levels. In most cases tested here, stimulation improved the performance of animals in the OF and EPM, displaying a clear anxiolytic effect [Figs. 4 and 5]. Stimulated animals explored significantly more OAs of the EFM and the central area of the OF than stressed animals, with levels comparable to those of the controls. SHAM group did not display these behaviors, suggesting that this was not the effect of surgical implantation of the electrodes. It is important to highlight that NPS, which achieved these robust effects, is low-frequency (only four pulses per second on average)

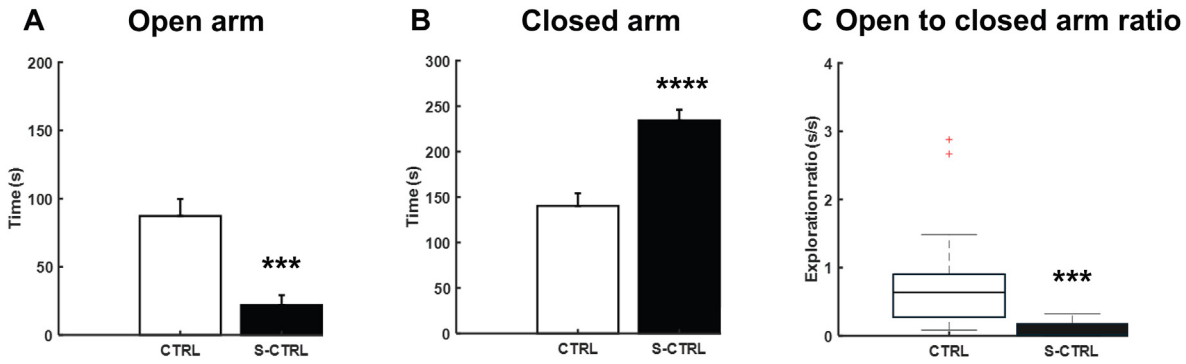


Fig. 2. Results of the EPM test to validate the stress protocol. (A) Exploration time of the open arm: animals in the S-CTRL group spent less time compared to the controls (S-CTRL: 22 ± 7 s *vs.* CTRL: 87 ± 13 s; ***$P < 0.001$, *t*-test). (B) S-CTRL animals spent more time exploring the closed arm than the controls (S-CTRL: 234 ± 12 s *vs.* CTRL: 140 ± 14 s; ****$P < 0.0001$, *t*-test). (C) The open *vs.* closed arm (OA/CA) exploration time ratio was lower for the S-CTRL group (S-CTRL: 0.08 [range 0–0.32] *vs.* CTRL: 0.64 [range 0.08–2.88]. Bar graphs are presented as mean ± standard error of the mean for consistency. Data are shown as mean ± standard error of the mean or median (range) for parametric and non-parametric data, respectively. ***$P < 0.001$, Mann–Whitney *U* test). CA: Closed arm; CTRL: Control; EPM: Elevated plus maze; OA: Open arm; S-CTRL: Stressed control.

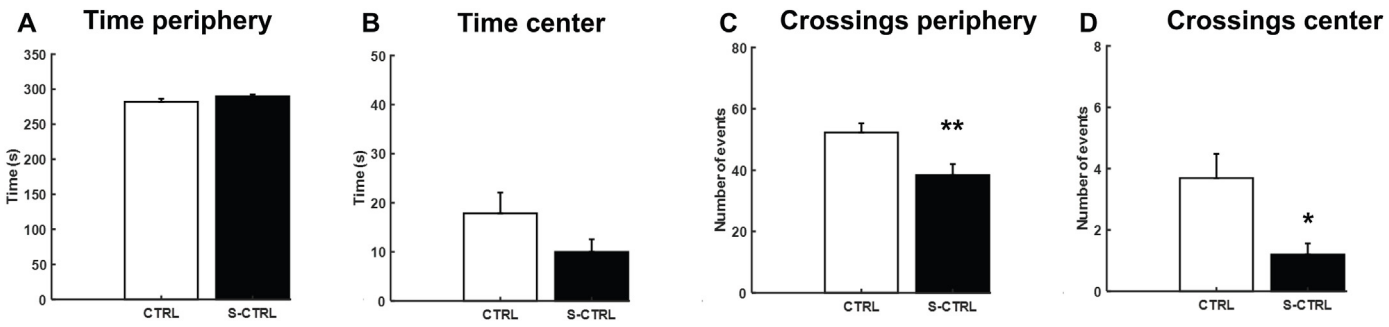


Fig. 3. Results of the OF test to validate the stress protocol. (A) No statistical difference was found between CTRL and S-CTRL animals in the time spent in the periphery of the open field arena (CTRL: 282 ± 4 s; S-CTRL: 290 ± 3 s; *t*-test). (B) There was no statistical difference between the groups in the time spent in the center of the arena (CTRL: 18 ± 4 s, S-CTRL: 10 ± 3 s; *t*-test). (C) S-CTRL animals displayed fewer quadrant crossings in the periphery (S-CTRL: 37 [range 18–57] *vs.* CTRL: 52 [range 34–66]; $^{**}P < 0.01$, Mann–Whitney *U* test). (D) Stressed animals displayed fewer quadrant crossings in the center (S-CTRL: 1 [range 0–3] *vs.* CTRL: 3 [range 0–8]. Bar graphs are presented as mean ± standard error of the mean for consistency. Data are shown as mean ± standard error of the mean or median (range) for parametric and non-parametric data, respectively. $^{*}P < 0.05$, Mann–Whitney *U* test). CTRL: Control; OF: Open field; S-CTRL: Stressed control.

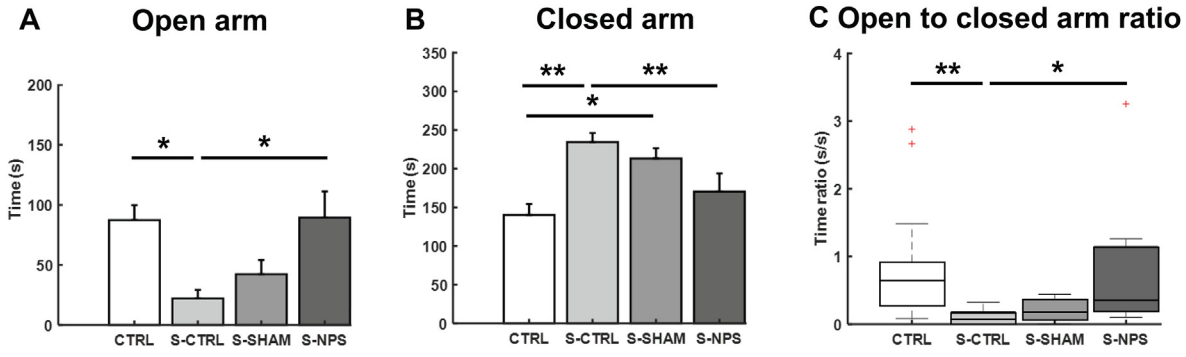


Fig. 4. Results of the EPM test for NPS effects. (A) In the open arm, stressed animals (S-CTRL) stayed significantly shorter than the controls (CTRL) and stimulated animals (S-NPS). Sham (S-SHAM) animals did not show significant differences from any other group (CTRL: 87 ± 13 s; S-CTRL: 22 ± 7 s; S-SHAM: 42 ± 12 s; S-NPS: 89 ± 22 s; $^{*}P < 0.05$, one-way ANOVA, Tukey's *post hoc* test). (B) In the closed arm, the S-CTRL group displayed longer duration in the closed arm than the CTRL and S-NPS groups. S-SHAM also displayed increased time in the closed arm than the CTRL group (CTRL: 140 ± 14 s; S-CTRL: 234 ± 12 s; S-SHAM: 213 ± 13 s; S-NPS: 170 ± 23 s; $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA, Tukey's *post hoc* test). (C) The S-CTRL group displayed lower open to closed arm (OA/CA) ratio compared to the CTRL and S-NPS groups (CTRL: 0.64 [range 0.08–2.88]; S-CTRL: 0.08 [range 0–0.32]; S-SHAM: 0.20 [range 0–0.44]; S-NPS: 0.36 [range 0.10–3.25]; $^{*}P < 0.05$ or $^{**}P < 0.01$, Kruskal–Wallis test, Dunn's *post hoc* test). Bar graphs are presented as mean ± standard error of the mean for consistency. Data are shown as mean ± standard error of the mean or median (range) for parametric and non-parametric data, respectively. ANOVA: Analysis of variance; CA: Closed arm; CTRL: Control; EPM: Elevated plus maze; NPS: Non-periodic stimulation; OA: Open arm; S-CTRL: Stressed control; S-NPS: Stressed and stimulated with non-periodic stimulation; S-SHAM: Stressed surgical control.

and thus a low-energy electrical stimulus method. It would be highly beneficial if such technologies were used in humans. Indeed, lower frequency stimuli mean that less energy is transferred from the power supply to the neural tissue, reducing the need for surgical intervention for battery change in the case of an internal pulse generator approach, as well as reducing the risk of lesions due to heat, electroporation, and other neurophysical factors at the electro-electrolyte interface.[30] Particularly, the specific value of four pulses was selected given that such value seems to be a natural "neural" resonance frequency for both epileptic and fear/anxiety phenomena.[41,56] Thus, applying a stimulus that has the same number of perturbations per second as the neural oscillation involved in the effect has cycles, but is delivered in a non-rhythmic fashion, can provide insight into the importance of the temporal pattern of stimulation and synchronization/desynchronization processes. Interestingly, literature also demonstrates that periodic (fixed-frequency) stimulation of the amygdala at 4 Hz facilitates seizures.[18] Moreover, designing and successfully testing a neuromodulation approach precisely engineered to tackle the central neurobiological mechanisms of brain function and dysfunction (in this case, neural synchronization) is superior to pure empiricism. It is also a strategy recommended by modern neuroengineering for safer, more efficient, and more efficacious treatments and/or electroceuticals.[11,15,28,57,58] We believe that NPS is an innovative therapeutic approach for treating pathological anxiety.

Overall, the anxiolytic effects of NPS were expected. The use of deep brain stimulation (DBS) and other non-invasive alternatives for the treatment of psychiatric disorders, including pathological anxiety, have been reported in literature.[59] Consistent with our findings, DBS has also been identified as a promising treatment option for patients with psychiatric disorders, such as anorexia nervosa, depression, and obsessive-compulsive disorder. According to Oudijn *et al*,[60] "the hypothesis is that DBS inhibits or functionally replaces the hyperactivity of the pathological network." Furthermore, the effect of NPS on the suppression of epilepsy has been demonstrated in extensive research.[17] Considering that pathological anxiety is highly comorbid with epilepsy, the dual therapeutic action of NPS was highly plausible. However, once again, while NPS has a low average frequency (four pulses per second), therapeutic DBS is usually delivered at 100 Hz or more.

There is now a good amount of evidence that NPS, like other spatiotemporally complex forms of brain stimulation, exerts its effects by means of beneficial modulation of neural synchronization. Considering the importance of amygdalar hyperexcitability and hypersynchronism across networks, it is plausible that the mechanisms underlying the therapeutic effect observed here are neural desynchronization or normalization of synchronization levels. However, there was no direct assessment capable of definitively testing this hypothesis, and, at the moment, such a framework is only speculative.

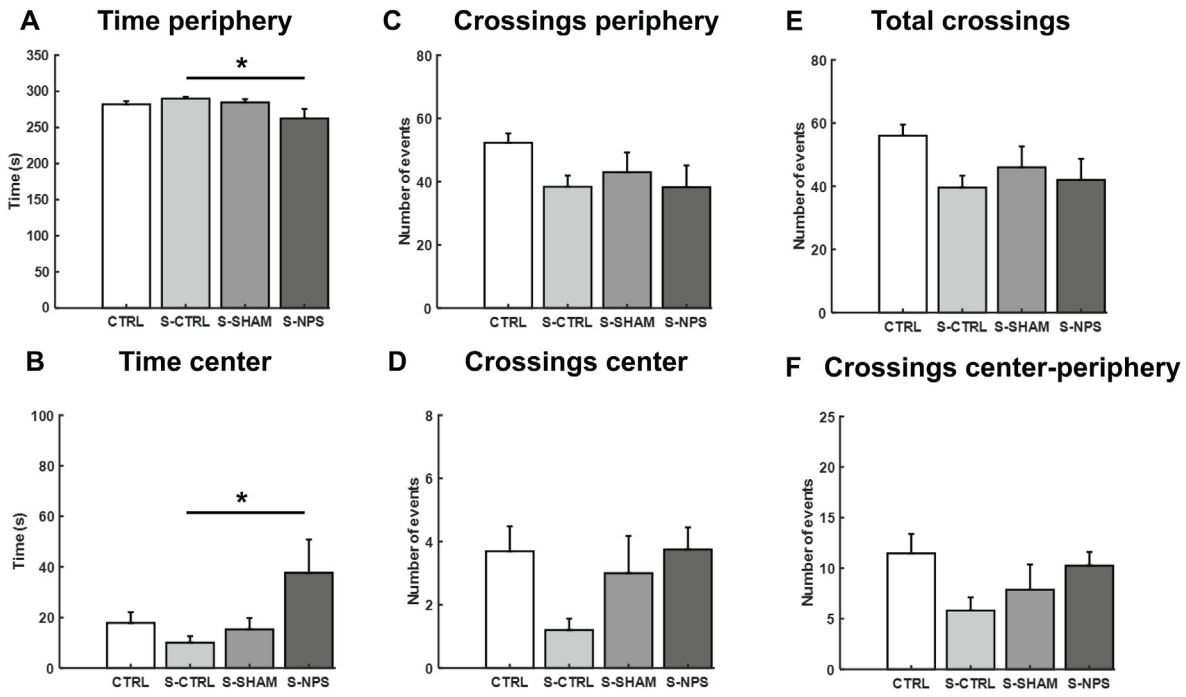


Fig. 5. Results of the OF test for NPS effects. Stimulated animals (S-NPS) spent a significantly lower time in the periphery (A. CTRL: 282 ± 4 s; S-CTRL: 290 ± 3 s; S-SHAM: 285 ± 5 s; S-NPS: 262 ± 13 s; *P <0.05, one-way ANOVA, Tukey's *post hoc* test) and increased time in the center (B. CTRL: 18 ± 4 s; S-CTRL: 10 ± 3 s; S-SHAM: 15 ± 5 s; S-NPS: 38 ± 13 s; *P <0.05, one-way ANOVA, Tukey's *post hoc* test) compared to stressed (S-CTRL) animals. No statistical differences were found among the groups regarding number of crossings in the periphery (C. CTRL: 52 [range 34–66]; S-CTRL: 37 [range 18–57]; S-SHAM: 37 [range 35–68]; S-NPS: 29.5 [range 19–75], Kruskal–Walis test, Dunn's *post hoc* test) or center (D. CTRL: 3 [range 0–8]; S-CTRL: 1 [range 0–3]; S-SHAM: 2 [range 0–8]; S-NPS: 4 [range 1–7], Kruskal–Walis test, Dunn's *post hoc* test). Additionally, there were no statistically significant differences in the number of total crossings (E. CTRL: 58 [range 36–72]; S-CTRL: 37.5 [range 18–60]; S-SHAM: 41 [range 19–69]; S-NPS: 33.5 [range 23–79], Kruskal–Walis test, Dunn's *post hoc* test) or the number of crossing in between zones, from center to periphery and vice-versa (F. CTRL: 13 [range 1–23]; S-CTRL: 5 [range 1–13]; S-SHAM: 7 [range 1–17]; S-NPS: 11 [range 5–15], Kruskal–Walis test, Dunn's *post hoc* test). Bar graphs are presented as mean ± standard error of the mean for consistency. Data are shown as mean ± standard error of the mean or median (range) for parametric and non-parametric data, respectively. ANOVA: Analysis of variance; CTRL: Control; NPS: Non-periodic stimulation; OF: Open field; S-CTRL: Stressed control; S-NPS; S-NPS: Stressed and stimulated with non-periodic stimulation; S-SHAM: Stressed surgical control.

Lesions in the basolateral nucleus of the amygdala can impair responses to new stimuli and emotional processing.[61] However, injury due to surgical implantation of the electrodes did not seem to have played a significant role in the present findings. Although there were no significant changes in S-SHAM animals compared to stimulated animals, there were also no differences compared to the controls. More importantly, most of the time values of the S-SHAM group were closer to the S-CTRL than the other way around. This lack of difference can also be attributed to an increase in variability in this group.

This study had some limitations. Although it is well established that the signals and symptoms observed in the EPM and OF tests are directly related to the substrates mediating anxiety levels, no direct observation of neural function was performed. Future work should address this by performing electrophysiological experiments on the underlying neural circuits, by neurochemical investigation of activation-related biomarkers (e.g., Fos proto-oncogene, AP-1 transcription factor subunit [cFos]), or by measuring physiological biomarkers of stress, such as blood levels of adrenocorticotropin and corticosterone. Another interesting aspect that should be explored in future research is the application of distinct temporal patterns of electrical stimulation to the amygdala to further understand the role of synchronization and desynchronization in the manifestation of the behaviors observed in this study. Finally, another limitation of our study was that the experiments were conducted only in male rats. It is well-documented that there are significant differences in anxiety-related health problems across sexes, notably in terms of prevalence, symptoms, and treatment responses.[62] Women are approximately twice as likely than men to be diagnosed with disorders such as generalized anxiety disorder, panic disorder, and specific phobias. This higher prevalence in women may be influenced by hormonal factors, genetic differences, or neurobiological variations. Hormonal fluctuations, particularly during menstrual cycle, pregnancy, and menopause, can exacerbate anxiety in women. Neurobiological differences, including variations in brain structure and neurotransmitter activity, also play a role in sexual disparities. Additionally, treatment responses vary, and women may respond better to selective serotonin reuptake inhibitors (SSRIs). NPS, as well as other forms of neurostimulation, may have distinct effects depending on the sex of the individual. Investigating the effects of NPS on anxiety-related symptoms across the gender dimension has the potential to not only provide further support for the method, but also clarify the mechanisms underlying the physiopathology of stress disorders/anxiety and its therapy.

Overall, the present findings suggested that NPS applied to the amygdala effectively suppresses the signals and symptoms of pathological anxiety, which in turn are successfully induced in non-stimulated animals. Although not directly tested, a previous understanding of the mechanisms of action of the method and the pathophysiology of the disorder suggests that the therapeutic effect was putatively obtained by desynchronization or normalization of synchronization of the amygdala circuitry underlying the function, whereas the possibility of being a byproduct of the lesion is unlikely. Finally, considering the fact that such a method did not result in impairment of basal amygdala function, as reported in previous works,[20] and its efficacy against epilepsy, a major comorbidity, NPS is a potential alternative for the treatment of dysfunctional anxiety in patients. NPS also has great innovative potential for novel neurotechnology, for its low-frequency (average) form of electrical stimulation and characteristics of precision-design neuroengineering.

Funding

This work was supported by the Fundação de Amparo à Pesquisa de Minas Gerais, Brazil (FAPEMIG) (No. APQ 02485–15) and the Conselho Narcional de Desenvolvimento Científico e Tecnológico (CNPq) (scholarship for scientific studies to the first author). The funding sources had no role in the study design; collection, analysis, and interpretation of data; writing of the report; or decision to submit the article for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors would like to thank Jasiara Carla de Olveira Coelho and Renato Marciano Maciel for their support during data collection; Márcio Flávio Dutra Moraes for wise advice on scientific matters; and Stéfano Pupe Johann for sharing the software used in tabulating times during the EPM and OF behavioral tests.

References

1. Jermakowicz WJ, Casagrande VA. Neural networks a century after Cajal. *Brain Res Rev*. 2007;55:264–284. https://doi.org/10.1016/j.brainresrev.2007.06.003.
2. von Stein A, Sarnthein J. Different frequencies for different scales of cortical integration: from local gamma to long range alpha/theta synchronization. *Int J Psychophysiol*. 2000;38:301–313. https://doi.org/10.1016/s0167-8760(00)00172-0.
3. Steriade M. The corticothalamic system in sleep. *Front Biosci*. 2003;8:d878–d899. https://doi.org/10.2741/1043.
4. Fuentealba P, Steriade M. The reticular nucleus revisited: Intrinsic and network properties of a thalamic pacemaker. *Prog Neurobiol*. 2005;75:125–141. https://doi.org/10.1016/j.pneurobio.2005.01.002.
5. Diekelmann S, Born J. The memory function of sleep. *Nat Rev Neurosci*. 2010;11:114–126. https://doi.org/10.1038/nrn2762.
6. Rezayat E, Clark K, Dehaqani MRA, Noudoost B. Dependence of working memory on coordinated activity across brain areas. *Front Syst Neurosci*. 2022;15:787316. https://doi.org/10.3389/fnsys.2021.787316.
7. Niebur E, Hsiao SS, Johnson KO. Synchrony: a neuronal mechanism for attentional selection? *Curr Opin Neurobiol*. 2002;12:190–194. https://doi.org/10.1016/S0959-4388(02)00310-0.
8. Guan A, Wang S, Huang A, et al. The role of gamma oscillations in central nervous system diseases: mechanism and treatment. *Front Cell Neurosci*. 2022;16:962957. https://doi.org/10.3389/fncel.2022.962957.
9. Palva JM, Palva S. Functional integration across oscillation frequencies by cross-frequency phase synchronization. *Eur J Neurosci*. 2018;48:2399–2406. https://doi.org/10.1111/ejn.13767.
10. Uhlhaas PJ, Singer W. Neural synchrony in brain disorders: relevance for cognitive dysfunctions and pathophysiology. *Neuron*. 2006;52:155–168. https://doi.org/10.1016/j.neuron.2006.09.020.
11. Famm K, Litt B, Tracey KJ, Boyden ES, Slaoui M. Drug discovery: a jump-start for electroceuticals. *Nature*. 2013;496:159–161. https://doi.org/10.1038/496159a.
12. Tass P. Resetting biological oscillators—a stochastic approach. *J Biol Phys*. 1996;22:27–64. https://doi.org/10.1007/BF00383820.
13. Tass PA. A model of desynchronizing deep brain stimulation with a demand-controlled coordinated reset of neural subpopulations. *Biol Cybern*. 2003;89:81–88. https://doi.org/10.1007/s00422-003-0425-7.
14. Reinhart RMG, Nguyen JA. Working memory revived in older adults by synchronizing rhythmic brain circuits. *Nat Neurosci*. 2019;22:820–827. https://doi.org/10.1038/s41593-019-0371-x.
15. Chiappalone M, Cota VR, Carè M, et al. Neuromorphic-based neuroprostheses for brain rewiring: state-of-the-art and perspectives in neuroengineering. *Brain Sci*. 2022;12:1578. https://doi.org/10.3390/brainsci12111578.
16. Carè M, Chiappalone M, Cota VR. Personalized strategies of neurostimulation: from static biomarkers to dynamic closed-loop assessment of neural function. *Front Neurosci*. 2024;18:1363128. https://doi.org/10.3389/fnins.2024.1363128.
17. Cota VR, de Oliveira JC, Damázio LCM, Moraes MFD. Nonperiodic stimulation for the treatment of refractory epilepsy: applications, mechanisms, and novel insights. *Epilepsy Behav*. 2021;121:106609. https://doi.org/10.1016/j.yebeh.2019.106609.
18. Cota VR, Medeiros Dde C, Vilela MRSDP, Doretto MC, Moraes MFD. Distinct patterns of electrical stimulation of the basolateral amygdala influence pentylenetetrazole seizure outcome. *Epilepsy Behav*. 2009;14(Suppl 1):26–31. https://doi.org/10.1016/j.yebeh.2008.09.006.
19. de Oliveira JC, Medeiros Dde C, de Souza E, Rezende GH, Moraes MFD, Cota VR. Temporally unstructured electrical stimulation to the amygdala suppresses behavioral chronic seizures of the pilocarpine animal model. *Epilepsy Behav*. 2014;36:159–164. https://doi.org/10.1016/j.yebeh.2014.05.005.
20. Réboli LA, Maciel RM, de Oliveira JC, Moraes MFD, Tilelli CQ, Cota VR. Persistence of neural function in animals submitted to seizure-suppressing scale-free nonperiodic electrical stimulation applied to the amygdala. *Behav Brain Res*. 2022;426:113843. https://doi.org/10.1016/j.bbr.2022.113843.
21. Mesquita MBS, Medeiros Dde C, Cota VR, Richardson MP, Williams S, Moraes MFD. Distinct temporal patterns of electrical stimulation influence neural recruitment during PTZ infusion: an fMRI study. *Prog Biophys Mol Biol*. 2011;105:109–118. https://doi.org/10.1016/j.pbiomolbio.2010.10.005.
22. Medeiros Dde C, Cota VR, Vilela MRSP, Mourão FAG, Massensini AR, Moraes MFD. Anatomically dependent anticonvulsant properties of temporally-coded electrical stimulation. *Epilepsy Behav*. 2012;23:294–297. https://doi.org/10.1016/j.yebeh.2012.01.004.
23. de Oliveira JC, Maciel RM, Moraes MFD, Rosa Cota V. Asynchronous, bilateral, and biphasic temporally unstructured electrical stimulation of amygdalae enhances the suppression of pentylenetetrazole-induced seizures in rats. *Epilepsy Res*. 2018;146:1–8. https://doi.org/10.1016/j.eplepsyres.2018.07.009.
24. de Souza Silva W, Maciel RM, Cota VR. Electrographic spectral signatures of animals submitted to pentylenetetrazole-induced seizures and treated with bilateral asynchronous non-periodic stimulation. In: Cota VR, Barone DAC, Dias DRC, Damázio LCM, eds. *Computational Neuroscience*. Cham: Springer International Publishing; 2019:258–266. https://doi.org/10.1007/978-3-030-36636-0_19.
25. Oliveira JPSe, Discacciati VRP, Medeiros DC, et al. In silico investigation of the effects of distinct temporal patterns of electrical stimulation to the amygdala using a network of Izhikevich neurons. In: de Almeida Ribeiro PR, Cota VR, Barone DAC, de Oliveira ACM, eds. *Computational Neuroscience*. Cham: Springer International Publishing; 2022:132–152. https://doi.org/10.1007/978-3-031-08443-0_9.
26. de Oliveira JC, Drabowski BMB, Rodrigues SMAF, Maciel RM, Moraes MFD, Cota VR. Seizure suppression by asynchronous non-periodic electrical stimulation of the amygdala is partially mediated by indirect desynchronization from nucleus accumbens. *Epilepsy Res*. 2019;154:107–115. https://doi.org/10.1016/j.eplepsyres.2019.05.009.
27. Batista Tsukahara VH, de Oliveira Júnior JN, de Oliveira Barth VB, de Oliveira JC, Rosa Cota V, Maciel CD. Data-driven network dynamical model of rat brains during acute ictogenesis. *Front Neural Circ*. 2022;16:747910. https://doi.org/10.3389/fncir.2022.747910.
28. Cota VR, Cançado SAV, Moraes MFD. On temporal scale-free non-periodic stimulation and its mechanisms as an infinite improbability drive of the brain's functional connectogram. *Front Neuroinf*. 2023;17:1173597. https://doi.org/10.3389/fninf.2023.1173597.
29. Medeiros DC, Cota VR, Oliveira ACP, Moreira FA, Moraes MFD. The endocannabinoid system activation as a neural network desynchronizing mediator for seizure suppression. *Front Behav Neurosci*. 2020;14:603245. https://doi.org/10.3389/fnbeh.2020.603245.
30. Cota VR, Drabowski BMB, de Oliveira JC, Moraes MFD. The epileptic amygdala: toward the development of a neural prosthesis by temporally coded electrical stimulation. *J Neurosci Res*. 2016;94:463–485. https://doi.org/10.1002/jnr.23741.
31. Avoli M, D'Antuono M, Louvel J, et al. Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro. *Prog Neurobiol*. 2002;68:167–207. https://doi.org/10.1016/s0301-0082(02)00077-1.
32. Panuccio G, D'Antuono M, de Guzman P, De Lannoy L, Biagini G, Avoli M. In vitro ictogenesis and parahippocampal networks in a rodent model of temporal lobe epilepsy. *Neurobiol Dis*. 2010;39:372–380. https://doi.org/10.1016/j.nbd.2010.05.003.
33. Prager EM, Aroniadou-Anderjaska V, Almeida-Suhett CP, Figueiredo TH, Apland JP, Braga MFM. Acetylcholinesterase inhibition in the basolateral amygdala plays a key role in the induction of status epilepticus after soman exposure. *Neurotoxicology*. 2013;38:84–90. https://doi.org/10.1016/j.neuro.2013.06.006.
34. Benini R, D'Antuono M, Pralong E, Avoli M. Involvement of amygdala networks in epileptiform synchronization in vitro. *Neuroscience*. 2003;120:75–84. https://doi.org/10.1016/s0306-4522(03)00262-8.
35. Imamura S, Tanaka S, Akaike K, Tojo H, Takigawa M, Kuratsu J. Hippocampal transection attenuates kainic acid-induced amygdalar seizures in rats. *Brain Res*. 2001;897:93–103. https://doi.org/10.1016/s0006-8993(01)02098-4.
36. Schramm J. Temporal lobe epilepsy surgery and the quest for optimal extent of resection: a review. *Epilepsia*. 2008;49:1296–1307. https://doi.org/10.1111/j.1528-1167.2008.01604.x.
37. Feindel W, Rasmussen T. Temporal lobectomy with amygdalectomy and minimal hippocampal resection: review of 100 cases. *Can J Neurol Sci*. 1991;18(4 Suppl):603–605. https://doi.org/10.1017/s0317167100032807.
38. Sah P, Faber ESL, Lopez De Armentia M, Power J. The amygdaloid complex: anatomy and physiology. *Physiol Rev*. 2003;83:803–834. https://doi.org/10.1152/physrev.00002.2003.
39. Davis M. The role of the amygdala in emotional learning. *Int Rev Neurobiol*. 1994;36:225–266. https://doi.org/10.1016/s0074-7742(08)60305-0.
40. Robinson OJ, Pike AC, Cornwell B, Grillon C. The translational neural circuitry of anxiety. *J Neurol Neurosurg Psychiatry*. 2019;90:1353–1360. https://doi.org/10.1136/jnnp-2019-321400.
41. Karalis N, Dejean C, Chaudun F, et al. 4-Hz oscillations synchronize prefrontal–amygdala circuits during fear behavior. *Nat Neurosci*. 2016;19:605–612. https://doi.org/10.1038/nn.4251.
42. Adhikari A, Topiwala MA, Gordon JA. Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety. *Neuron*. 2010;65:257–269. https://doi.org/10.1016/j.neuron.2009.12.002.

43. Lesting J, Geiger M, Narayanan RT, Pape HC, Seidenbecher T. Impaired extinction of fear and maintained amygdala-hippocampal theta synchrony in a mouse model of temporal lobe epilepsy. *Epilepsia*. 2011;52:337–346. https://doi.org/10.1111/j.1528-1167.2010.02758.x.
44. Diamond DM, Zoladz PR. Dysfunctional or hyperfunctional? The amygdala in posttraumatic stress disorder is the bull in the evolutionary China shop. *J Neurosci Res*. 2016;94:437–444. https://doi.org/10.1002/jnr.23684.
45. Yilmazer-Hanke D, O'Loughlin E, McDermott K. Contribution of amygdala pathology to comorbid emotional disturbances in temporal lobe epilepsy. *J Neurosci Res*. 2016;94:486–503. https://doi.org/10.1002/jnr.23689.
46. Prager EM, Aroniadou-Anderjaska V, Almeida-Suhett CP, et al. The recovery of acetylcholinesterase activity and the progression of neuropathological and pathophysiological alterations in the rat basolateral amygdala after soman-induced status epilepticus: relation to anxiety-like behavior. *Neuropharmacology*. 2014;81:64–74. https://doi.org/10.1016/j.neuropharm.2014.01.035.
47. Milad MR, Rauch SL, Pitman RK, Quirk GJ. Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biol Psychol*. 2006;73:61–71. https://doi.org/10.1016/j.biopsycho.2006.01.008.
48. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 1998.
49. McDonald AJ. Functional neuroanatomy of the basolateral amygdala: neurons, neurotransmitters, and circuits. *Handb Behav Neurobiol*. 2020;26:1–38. https://doi.org/10.1016/b978-0-12-815134-1.00001-5.
50. Gameiro GH, Gameiro PH, Andrade Ada S, et al. Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress. *Physiol Behav*. 2006;87:643–649. https://doi.org/10.1016/j.physbeh.2005.12.007.
51. Padovan CM, Guimarães FS. Restraint-induced hypoactivity in an elevated plus-maze. *Braz J Med Biol Res*. 2000;33:79–83. https://doi.org/10.1590/s0100-879x2000000100011.
52. Ramirez S, Liu X, MacDonald CJ, et al. Activating positive memory engrams suppresses depression-like behaviour. *Nature*. 2015;522:335–339. https://doi.org/10.1038/nature14514.
53. Buccafusco JJ. The revival of scopolamine reversal for the assessment of cognition-enhancing drugs. In: Buccafusco JJ, ed. *Methods of Behavior Analysis in Neuroscience*. second ed. Boca Raton: CRC Press/Taylor & Francis; 2008:329–343. https://doi.org/10.1201/NOE1420052343.ch17.
54. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacol*. 1987;92:180–185. https://doi.org/10.1007/BF00177912.
55. Silveira PP, Portella AK, Clemente Z, Gamaro GD, Dalmaz C. The effect of neonatal handling on adult feeding behavior is not an anxiety-like behavior. *Int J Dev Neurosci*. 2005;23:93–99. https://doi.org/10.1016/j.ijdevneu.2004.07.018.
56. Moraes MFD, Mishra PK, Jobe PC, Garcia-Cairasco N. An electrographic analysis of the synchronous discharge patterns of GEPR-9s generalized seizures. *Brain Res*. 2005;1046:1–9. https://doi.org/10.1016/j.brainres.2005.03.035.
57. Sunderam S, Gluckman B, Reato D, Bikson M. Toward rational design of electrical stimulation strategies for epilepsy control. *Epilepsy Behav*. 2010;17:6–22. https://doi.org/10.1016/j.yebeh.2009.10.017.
58. Cota VR, Moraes MFD. Editorial: engineered neuromodulation approaches to treat neurological disorders. *Front Neurosci*. 2022;16:1038215. https://doi.org/10.3389/fnins.2022.1038215.
59. Cheng YC, Kuo PH, Su MI, Huang WL. The efficacy of non-invasive, non-convulsive electrical neuromodulation on depression, anxiety and sleep disturbance: a systematic review and meta-analysis. *Psychol Med*. 2022;52:801–812. https://doi.org/10.1017/S0033291721005560.
60. Oudijn MS, Storosum JG, Nelis E, Denys D. Is deep brain stimulation a treatment option for anorexia nervosa? *BMC Psychiatr*. 2013;13:277. https://doi.org/10.1186/1471-244X-13-277.
61. LeDoux JE. Emotion and the amygdala. In: Aggleton JP, ed. *The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction*. New York: Wiley-Liss; 1992:339–351.
62. Park HY. Sex/gender differences in depression and anxiety disorders. In: Kim N, ed. *Sex/gender-specific Medicine in Clinical Areas*. Singapore: Springer Nature; 2024:369–379.