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Seizure suppression by asynchronous non-periodic electrical stimulation of the amygdala is partially mediated by indirect desynchronization from nucleus accumbens

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ABSTRACT

Electrical stimulation (ES) of the nervous system is a promising alternative for the treatment of refractory epilepsy. Based on the understanding that seizures are the expression of neural hypersynchronism, our group developed and tested a non-standard form of low-energy temporally unstructured ES termed NPS (Non-periodic stimulation), with pseudo-randomized inter-pulse intervals. Previous investigation demonstrated that NPS applied to the amygdala has a robust anticonvulsant effect against both acute and chronic seizures, and suggested that its therapeutic effect is based on direct desynchronization of ictogenic neural circuits. Further mechanistic investigation using functional magnetic resonance imaging has shown that NPS also activates nucleus accumbens (NAc) in seizure-free rats, raising the hypothesis of an alternative therapeutic mechanism: NPS-enhanced indirect inhibition / desynchronization of ictogenic circuits by NAc. In order to investigate this idea, here we evaluated behavior and cortical electrographic activity from animals submitted to pentylenetetrazole (PTZ) induced seizures, treated with NPS and with or without bilateral electrolytic lesion of NAc. NPS-treated animals with bilateral lesion of NAc expressed unexpected straub tail in addition to other stereotypical convulsive behavior, displayed increased susceptibility to PTZ (lower drug threshold), and had a much longer electrographic seizure, with a greater number of spikes, firing at a higher rate. Moreover, analysis of spike morphology showed an increase in amplitude and slope in these animals, suggesting that ablation of NAc results in disinhibition and/or increase of neural synchronism within ictogenic circuits. NPS had no therapeutic effect whatsoever in lesioned animals, while it displayed a mild anticonvulsant effect in those with intact brains. Results corroborate the notion that NAc has a key role in controlling aberrant epileptiform activity in ictogenic circuits through indirect polysynaptic connections that may enroll the ventral pallidum and ventral tegmental area. They also point to the possibility that NPS may enhance this effect, putatively by benefiting from the structure's property of detecting saliences.

1. Introduction

Epilepsy is a neurological disorder characterized by recurring and spontaneous seizures, which are episodes of aberrantly intense neural activity (Fisher et al., 2005). It affects circa 65 million individuals worldwide (Thurman et al., 2011) and is regarded as a public health issue, given its biological, social, and economic impact (Harden et al., 2007). Current first-choice treatment in medical practice are pharmacotherapy (Perucca and Tomson, 2011) and surgical ablation of epileptogenic focus (Cascino, 2004). Although both modalities are reasonably efficacious, a considerable portion of patients do not attain full control of their seizures with antiepileptic drugs (French, 2007), nor they can be submitted to surgery mainly due to the absence of a well-defined target (Spencer, 2002). Novel therapeutic approaches are in obvious need.

The unbalance between neural excitation and inhibition – in favor

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of the former – due to several molecular and cellular factors is largely understood as a fundamental mechanism underlying the pathophysiology of epilepsy, with seizures resulting from a permanent state of hyperexcitability of neural tissue (Fisher et al., 2005). On the other hand, there is now mounting evidence suggesting hypersynchronism is also a key factor for understanding the disease and for enabling robust, safe, and efficacious treatment (Uhlhaas and Singer, 2006; Medeiros et al., 2014; Cota et al., 2016). For the sake of clarity, synchronism can be defined as the relation between dynamics of two coupled oscillatory systems (Kreuz et al., 2007). Thus, if the oscillatory activity of a given neural circuit drives the dynamics of a second one, it is said that they are synchronized and some form of synchronization may be objectively observable in their temporal characteristics.

While decreased synchronization may contribute to functional deafferentation of neural networks, sufficient synchronization dynamically connects areas and networks for myriad brain functions, such as sleep (Steriade et al., 1993; Steriade, 1993) and memory processing (Born and Wilhelm, 2012). By the same token, in epilepsy, hypersynchronization contributes to the aberrant coupling of dysfunctional hyperactive micro-oscillators giving rise to ictogenesis (Paz and Huguenard, 2015; Varela et al., 2001; Uhlhaas and Singer, 2006), while desynchronization induces functional isolation that putatively impairs epileptic phenomena (Medeiros et al., 2014; Cota et al., 2016). This relation between the underpinnings of epilepsy and neural synchronization has been reported in many in vivo (Imamura et al., 2001; Lesting et al., 2011), in vitro (Benini et al., 2003; Koganezawa et al., 2008), and *in silico* studies (Abeles et al., 2004; Tass and Hauptmann, 2007), even though the process seems to be more intricate than what was first believed, once both synchronization and desynchronization may be in place in the beginning and in the propagation of epileptic seizures (Jiruska et al., 2013; Zhang et al., 2016).

Based on the understanding that epilepsy is a dysfunction of neural (mostly hyper) synchronism, our group believes that approaching both ictogenesis and epileptogenesis in the network level and impairing circuit hypersynchronization is a valid – maybe even superior – means to treat epilepsy (Cota et al., 2016). With that in mind, in 2009 we successfully tested the hypothesis that electrical stimulation (ES), even with a low count of pulses per second, applied to a neural substrate considered to play a key role in dysfunctional synchronization (the basolateral amygdala – AMY) would differentially modulate the expression of convulsive behavior according to only the temporal structure (or lack thereof) of stimuli: periodic (PS – periodic stimulation) with fixed interval between pulses (IPI – inter-pulse intervals) or; non periodic (NPS – non-periodic stimulation) with pseudo-randomized IPI. Our study with animals submitted to a controlled infusion of convulsant drug pentylenetetrazole (PTZ) has shown that while PS had a convulsant effect decreasing drug threshold to elicit convulsive behavior, NPS displayed an anticonvulsant effect almost doubling the amount of drug necessary to evoke generalized tonic-clonic seizures (GTCS) (Cota et al., 2009). Further investigation showed that NPS can also abate spontaneous and recurrent seizures of the late phase (45 days after induction of status epilepticus) of the pilocarpine model of temporal lobe epilepsy, reducing both their number and duration, as major evidence that its therapeutic mechanisms are preserved in dysfunctional tissue (Oliveira et al., 2014). Finally, recent investigation demonstrated that anticonvulsant power of NPS gradually increases when novel spatial and temporal parameters are incorporated, making biphasic, bilateral, and interhemispheric asynchronous NPS the most efficacious protocol (Oliveira et al., 2018).

Although these studies are evidence that neural desynchronization may be an important factor of seizure suppression, a more mechanistic investigation using non-invasive imaging protocols was carried out to better understand if that was, in fact, the case with NPS. fMRI images from anesthetized animals obtained during the administration of PTZ showed that PS applied to the basolateral amygdala increases activation of structures across the ipsilateral limbic system axis, while NPS promotes local inhibition with contralateral activation, corroborating the notion of synchronization and desynchronization as key mechanisms of ictogenesis and its abatement, respectively (Mesquita et al., 2011). Of particular interest, this study also showed that NPS applied to animals without the administration of PTZ induces increases of fMRI signal in frontal structures, with a special effect in the nucleus accumbens (NAc), bilaterally.

This unexpected finding has raised the hypothesis that NPS may also wield its therapeutic effect indirectly through the activation of NAc which, by its turn, would modulate, inhibiting or desynchronizing neural networks participating in ictogenesis, such as those in the mesial temporal lobe. In fact, other studies suggest that activation of NAc may have a beneficial modulating effect on such circuits. Russo and Nestler (2013) showed that NAc connects to the amygdala and hippocampus through the ventral tegmental area (VTA) and Kowski et al. (2015) demonstrated that stimulation of NAc reduces seizure frequency in patients with focal epilepsy.

In this present study, we tested the hypothesis that nucleus accumbens (NAc) has an important role in the suppression of seizures by NPS, with a putative inhibitory and/or desynchronizing effect over ictogenic neural circuits. For this, we applied NPS to rats submitted to acute ictogenesis induced by controlled intravenous infusion of PTZ, with or without bilateral electrolytic lesion of NAc. By assessing occurrence and characteristics of convulsive behavior, we were able to verify the valence (convulsant versus anticonvulsant) of the participation of NAc in seizures. In order to determine the effect of both NPS and NAc lesions in neural synchronism, electrophysiological recordings of the cortical surface were also performed and overall features of electrographic seizures were compared between groups. Finally, the morphology of epileptiform spikes, a high amplitude fast transient change of voltage in the recording, was also investigated, once these electrographic events and their features are correlated with synchronization / desynchronization levels of neuronal populations (Wu et al., 2013; Niedermeyer, 2005; Nunez and Srinivasan, 2005).

2. Material and methods

2.1. Animals and groups

All experiments were done in accordance with the Ethics Committee on Research Involving Animals (Comitê de Ética no Uso de Animais de Laboratório – CEUA) of the Federal University of São João Del Rei (UFSJ) (Protocol 13/2013). The procedures are in full accordance with international guidelines for the care of animals in research. During the experiments, the animals were maintained in a light-dark cycle of 12 h (lights on at 7 a.m. and off at 7 p.m.), at room temperature, water and food ad libitum.

Male Wistar rats (n = 26; weighing 230–330 g) were randomly distributed among three experimental groups: control group (n = 12), NPS group (n = 8), and NPSNAc- group (n = 6). All animals were submitted to acute seizures induced by controlled infusion of the convulsant agent pentylenotetrazole (PTZ), recording of electroencephalographic cortical activity, and behavioral evaluation. Each group underwent a specific experimental protocol: 1) CTRL group: administration of PTZ, without electrical stimulation (ES) - NAc preserved 2) NPS group: administration of PTZ, application of ES (NPS), and NAc preserved and; 3) NPSNAc- group: administration of PTZ, application of ES (NPS), and bilateral electrolytic lesion of NAc.

2.2. Surgical procedures

All animals, including controls, underwent stereotaxic surgery for implantation of depth ES electrodes in the right and left amygdala, and a superficial electrode for recording of the cortex (CX). Rats were anesthetized with ketamine 100 mg / kg (50 mg / ml – Konig from Brazil, Santana do Paraíba, SP, Brazil), xylazine 5.0 mg / kg (20 mg / ml –

Syntec from Brazil, Cotia, SP, Brazil), and fentanyl 0.025 mg / kg (50 mcg / ml - Union Chemical from Brazil, Londrina, PR, Brazil), and positioned in a stereotaxic frame (Insight Equipments Ltda, Ribeirão Preto, SP, Brazil). Bipolar electrodes (a twisted pair with tip separation 0.5 mm) made of stainless steel Teflon®-coated wires (diameter 127 µm, model #791400, A–MSystems Inc., California, USA) were implanted bilaterally in the amygdala at coordinates: AP = 2.8 mm, ML = ± 5.0 mm from Bregma and 7.2 mm from Dura mater (Paxinos and Watson, 1998). Two microsurgical-screws (length 4.7 mm, diameter 1.17 mm, Fine Science Tools, Inc., North Vancouver, Canada) were also implanted, one for cortical recording in the right hemisphere and other used as a reference in the frontal bone. In addition, a structural microsurgical screw was placed in the left parietal bone. The electrodes were fixed to the bone with zinc cement and soldered to a phone jack (type RJ-11), which was fixed onto the skull with dental acrylic.

2.3. PTZ infusion

After 7 days of surgical recovery, the animals underwent intravenous infusion of PTZ (10 mg/ml – Sigma-Aldrich, diluted in saline 0.9%) by means of a catheter (BD Angiocath Catheter I.V. – 24GA × 0.75IN – 0.7 × 19 mm; Becton-Dickinson Industrial, Juiz de Fora, MG, Brazil) in the lateral caudal vein. The catheter was connected, through a cannula, to an infusion pump set at a rate of 0.4 ml/min. Infusion of PTZ started simultaneously to ES (NPS pattern) according to the experimental group (CTRL: no stimulation, NPS and NPSNAc-: with stimulation). The behavioral effect of treatment with or without electrolytic lesion of NAc was assessed by calculation of PTZ threshold: the amount of injected drug normalized to body weight necessary to induce generalized tonic-clonic seizures (GTCS). The amount of drug injected was calculated by the rate of infusion multiplied by the latency (time from the beginning of the infusion to trigger each behavior). PTZ infusion was terminated immediately after animals started GTCS.

2.4. Electrical stimulation

For electrical stimulation (ES) of the bilateral amygdala, we used non-periodic temporal pattern (NPS) (Fig. 1A) with random intervals between pulses at a fixed amount of 4 pulses per second (low-frequency ES on average; see Cota et al., 2009 for further details). Our research group designed and built an electrical stimulator composed of a constant-voltage isolation unit driven by the output of an MP3 player (model NWZ-B152 26B, Sony) (Medeiros et al., 2012). Pulses were biphasic, square waves of 100 µs of duration with variable amplitude between 100 and 600 µA, according to the susceptibility of each animal. Current amplitudes were measured by a shunt resistor in series with each animal's brain. The current threshold susceptibility test was performed the day before the experiment and started with 50 µA and gradually increased by 50 µA steps until the first behavioral response. From the onset of behavioral response, the current was then kept at the highest level that did not produce it. Pulses were delivered bilaterally and asynchronously between the hemispheres (Fig. 1B). Stimulation started simultaneously to the infusion of PTZ and, similarly, was terminated immediately after animals displayed GTCS.

2.5. Bilateral lesion of nucleus accumbens

The electrolytic lesion of bilateral NAc was carried out using bipolar electrodes (stereotaxic coordinates: AP: +2.2 mm, ML: ± 0.9 mm, DV: 7.0 mm) (Paxinos and Watson, 1998) during the surgical procedure, after implantation of recording and ES electrodes in the target substrates. The electrolytic lesion was carried out by applying direct current, of approximately 1 mA amplitude, for 10 s, according to other NAc lesion protocols (Gal et al., 1997; Kelsey et al., 2009). The lesion apparatus consisted simply of a 9 V battery and an on / off switch. Panel C of Fig. 1 shows the current applied and histological findings of the lesion area obtained by the procedure: the black area shows the smallest lesion and gray area shows the largest lesion.

2.6. Video-EEG recording

Neural synchronization phenomena can manifest in many different ways (Lehnertz et al., 2009). A useful tool to investigate it is electroencephalography (EEG), due to its high temporal resolution. Therefore, electrographic recordings were obtained from all animals by means of an electrode positioned on the surface of the cortex.

The EEG signal was pre-amplified by a built-in headstage with a

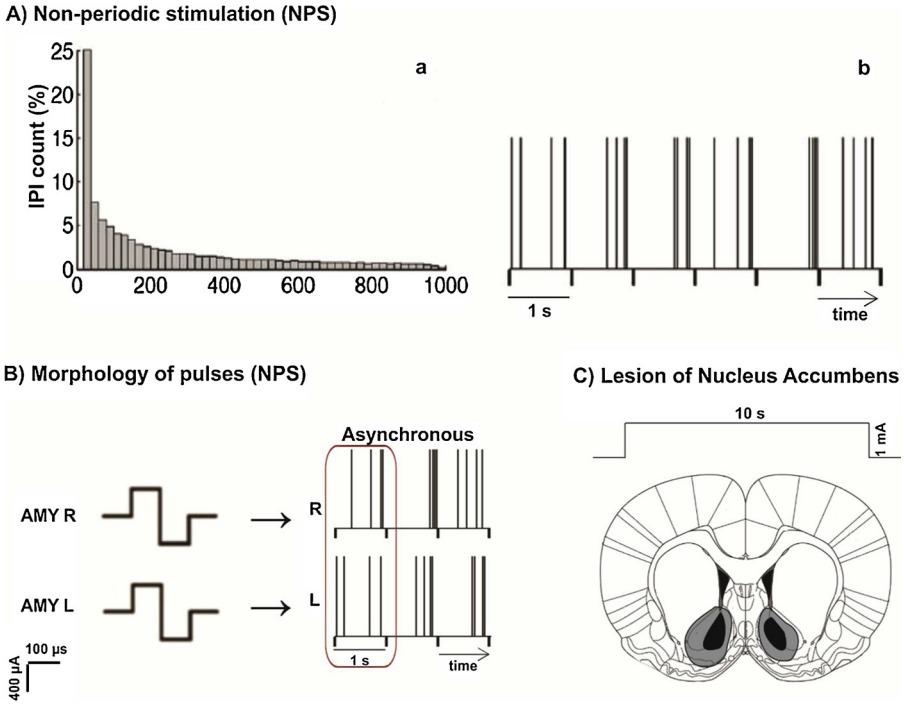

Fig. 1. A) Temporal pattern of non-periodic stimulation (NPS). Histogram of intervals between pulses (IPI) (a); an example of the temporal distribution of pulses in time (4 pulses per sec) (b). B) Morphology of biphasic pulses applied bilaterally and asynchronously. C) Morphology of current applied to NAc in order to induce electrolytic lesion (top) and lesion areas: smallest (black spot) and largest (gray spot) areas (bottom).

5 V/V differential gain (Cota et al., 2010), amplified (2000 V/V) and filtered (0.3 Hz high pass, 300 Hz low pass) by an analog commercial amplifier (model # 3500, A–M Systems, Sequim, WA, USA). Subsequently, the signal was digitized using 12 bits of resolution at a sampling frequency of 1000 Hz, using an analog-to-digital converter (NI-DAQ 6023E model) from National Instruments (Austin, Texas, USA) controlled by a LabView® virtual instrument developed in our lab.

To evaluate the effect of the experimental procedures, animal behavior was recorded in video simultaneously to voltage recordings in a video-EEG system. The animal was placed in free motion in a chamber positioned inside a Faraday cage (for elimination of electromagnetic noise) and its behavior was filmed in multifocal CCD camera. Video signal was captured by a Pinnacle capture system (USB 710) and sent to the computer. The composite image of video and EEG was then sent via a video board to a DVD recorder.

2.7. Spike detection and characterization

Epileptiform spikes are a result of synchronous neural discharges and features of their morphology can provide insights on the level of synchronization (Wu et al., 2013; Niedermeyer, 2005; Nunez and Srinivasan, 2005). In this work, spike activity and morphology were measured in order to assess the influence of NPS and NAc lesion on neural activity.

Raw EEG was pre-processed in order to highlight spikes by band-pass filtering the signal at 10 to 100 Hz. Due to contamination from ES in two out of the three groups, the period of an electrographic seizure was considered to begin with the behavioral onset of GTCS, when PTZ infusion and NPS were both interrupted, and finish with the termination of epileptiform activity. Termination of epileptiform activity was visually identified in all animals, based on the following objective criteria: 1) a spike is a transient change of voltage on the tracing, distinguishable in amplitude from basal activity, lasting a maximum of 70 ms (IFSENC, 1974); 2) an electrographic seizure is composed of multiple spikes at a minimum firing rate of one per second. This visual inspection of EEG was performed blindly in reference to the group by two independent and experienced researchers from our lab and their results coincided virtually perfectly. Duration of epileptiform activity for each group was, thus, computed.

Spikes within the electrographic seizure (only) were then detected by a threshold crossing algorithm with restrictions (such as in Maccione et al., 2009 or in Medeiros et al., 2014). Threshold level was defined as a fixed percentage for the whole recording, but applied to the maximum value of each 1 s time window, so as to coup with the high variability of spike amplitudes across a single seizure. The percentage values were subjectively defined for each animal in order to obtain the minimum number of false positives and varied from 60% to 90%. Mean percentage values did not significantly differ between experimental groups (CTRL: 70 ± 0%; NPS: 67 ± 5% and; NPSNAc-: 70 ± 0%). A spike was detected every time an upward threshold crossing occurred immediately followed by a downward crossing and its timestamp was defined as the time instant of the peak between crossings. The following restrictions were applied: minimum absolute value of threshold at 225 μV and minimum distance between spikes of 100 ms (Fig. 2).

After detection, the total number of spikes and mean firing rate (total number divided by seizure duration) were computed. Also, spike amplitude of the positive phase and upward slope were calculated. Amplitude was simply the absolute voltage value of the spike peak. Slope was calculated by dividing the subtraction of voltage values (Δy) by the subtraction of timestamps (Δx) of the spike peak and its immediately preceding valley (or minimum) (Fig. 2 Detail).

2.8. Histology

After the experimental procedure, the animals' brains were extracted for preservation and posterior histological processing (coronal sections of 40 μm thickness) by cresyl violet or neutral red (2%) staining techniques to verify the positioning of electrodes. All animals in this study had histological confirmation of electrode position (Supplementary Figs. 1 and 2).

2.9. Statistical analysis

Data are presented as means ± S.E.M. Statistical comparisons were made using one-way ANOVA and post hoc by Tukey's multiple comparisons of group pairs. Values of $p < 0.05$ were considered statistically significant.

3. Results

All animals in this study displayed a sequence of behaviors directly related to the gradual recruitment of neural substrates, from local to generalized, which is stereotypical of the model of acute seizures induced by controlled infusion of PTZ. First, animals expressed facial automatism followed by myoclonic jerks. Seizures then progressed to forelimb clonus (FC) and generalized tonic-clonic seizures (GTCS). Differently from other groups, animals of the NPSNAc- group continue to display erratic convulsive behavior, such as forelimb and hindlimb clonus much after GTCS has ended. Moreover, 4 out of 6 animals of the NPSNAc- group also displayed straub tail, which is unexpected of the model used, in the meantime between expression of partial and generalized seizing behaviors. The occurrence of this behavior was significantly increased in this group ($p < 0.05$; Fisher's Exact test; Table 1)

PTZ threshold to elicit GTCS was found to be significantly lower for the NPSNAc- group when compared to controls ($p < 0.05$, one-way Anova), while no other pair of groups displayed statistically significant differences (Fig. 3).

Not all animals displayed an artifact-free electrographic recording, due mainly to contamination related to intense movement during seizures and / or to the connection with ES apparatus. Thus, the sample size is different for electrophysiological results: CTRL, $n = 9$; NPS, $n = 7$ and; NPSNAc-, $n = 5$.

Visual inspection of raw EEG tracing during seizures shows two distinct phases for the CTRL and NPS groups: tonic phase with high-frequency oscillations and sparse spikes and the clonic phase with high amplitude sustained polyspike activity (Fig. 4 – top/black and middle/blue tracings). NPSNAc- group electrographic activity did not show these two phases clearly, but actually clusters of polyspikes and intense interictal activity from the beginning (Fig. 4 – bottom/red tracing). Also, epileptiform activity in NPSNAc- recurred several times after the first one with a few seconds between episodes (see, for instance, clear epileptiform activity by the end of NPSNAc- tracing in the bottom of Fig. 4). In fact, total duration of electrographic activity was significantly much longer in lesioned animals when compared to both CTRL ($p < 0.01$) and NPS ($p < 0.001$) groups. NPS animals showed only a not significant trend ($p < 0.06$) to a decreased duration in relation to controls (Fig. 5).

Although with somehow different overall morphologies, spikes were easily detected in all EEG tracings of all groups (Fig. 4 – details). Fig. 6 shows the mean spike (solid line) and the standard deviation (shadow) in an 80 ms-long time window for each experimental group.

Total number of spikes was significantly greater in the NPSNAc- group when compared to both CTRL ($p < 0.001$) and NPS ($p < 0.0001$). Similarly, as in the case of the duration of epileptiform activity, there was a trend to a decrease in the number of spikes in the NPS group when compared to CTRL ($p < 0.06$) (Fig. 7).

Parameters of spike morphology were also different between groups. Mean firing rate in the NPSNAc- was significantly higher when compared to NPS group ($p < 0.05$) (Fig. 8, panel A). Moreover, spike amplitude was greater in the NPSNAc- group when compared to CTRL ($p < 0.05$) (Fig. 8, panel B). Finally, spike slope was greater in

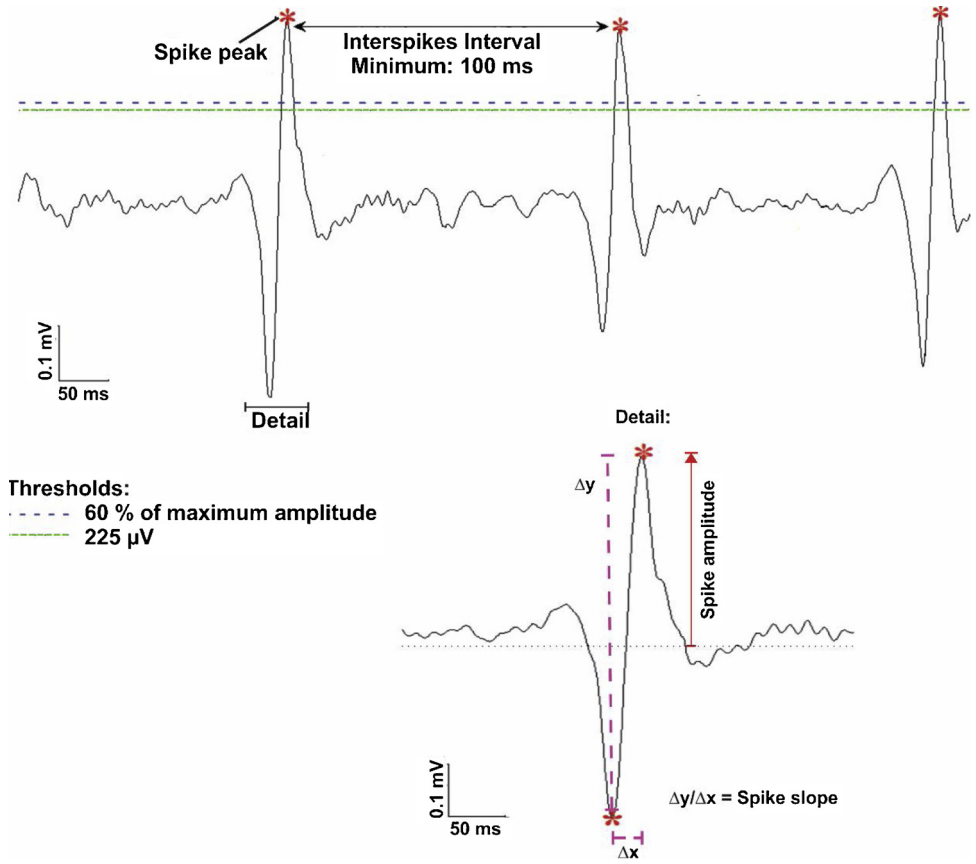

Fig. 2. Detection of epileptiform spikes and characterization of spike morphology. Top EEG tracing shows thresholds for detection of spikes, identification of peaks (red stars), and intervals between spikes. Detail in the bottom shows the calculation of spike amplitude and slope.

Table 1
Contingency table for the occurrence of straub tail. Animals in the NPSNAc- group significantly displayed a greater number of occurrences (*p < 0.05, Fisher's Exact test).

	no straub tail	straub tail
CTRL	12	0
NPS	8	0
NPSNAc-	2	4*

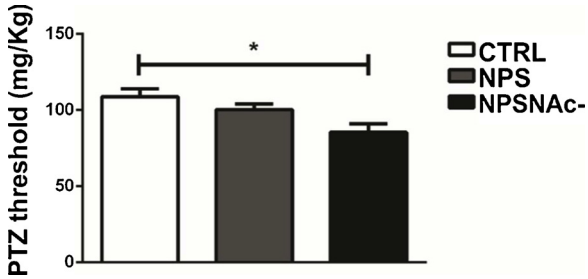

Fig. 3. PTZ threshold to elicit GTCS. NPSNAc- group displayed a significantly smaller drug threshold when compared to CTRL ($p < 0.05$, one-way Anova).

NPSNAc- when compared to both CTRL ($p < 0.0001$) and NPS ($p < 0.0001$) (Fig. 8, panel C).

4. Discussion

In this work, we studied the effects of electrolytic lesion of NAc, bilaterally, in the behavior and in the cortical electrographic activity of animals submitted to acute PTZ-induced seizures treated with NPS. The goal was to test the hypothesis that this structure has a role in impairing ictogenesis after application of NPS in the amygdala, such as suggested in previous work of the group (Mesquita et al., 2011). Our hypothesis was that NAc would exert a desynchronizing effect on ictogenic circuits that may involve the limbic system, the neocortex, and even mid and hindbrain areas, through polysynaptic modulatory connections (Cota et al., 2016).

Although they are not enough for an accurate neuroanatomical description, present behavioral results corroborate this notion of a desynchronizing effect towards key ictogenic networks. For instance, straub tail has been described as a behavior resulting from the activation of the opioidergic system encompassing neocortex and areas within the mesial temporal lobe (e.g. hippocampus) (Fonck et al., 2003). By this token, the significant occurrence of straub tail in NPSNAc- group suggests stronger recruitment of such ictogenic circuits, although the complexity of such neurotransmitter system jeopardizes any definite conclusions. Additionally, the decreased PTZ threshold to evoke GTCS in the same group shows that seizures originating from broader circuits encompassing not only prosencephalon, but also mid and hindbrain structures, were facilitated in these animals. It is important to highlight that these animals were also submitted to the application of NPS to the amygdala, which has been shown to have a robust anticonvulsant effect in several other studies of our group (Cota et al., 2009; Medeiros et al., 2012; Oliveira et al., 2014, 2018). Taken together, this evidence indicates that the ablation of NAc not only impairs the therapeutic effect of NPS, but also worsens partial or generalized seizures. Hence, NAc is probably important in controlling aberrant activity in both mesial temporal lobe or in more spread circuitry, and this effect is putatively enhanced by the application of NPS to the amygdala.

This understanding is also corroborated by electrophysiology results. Electrographic seizures lasted much longer in animals of the

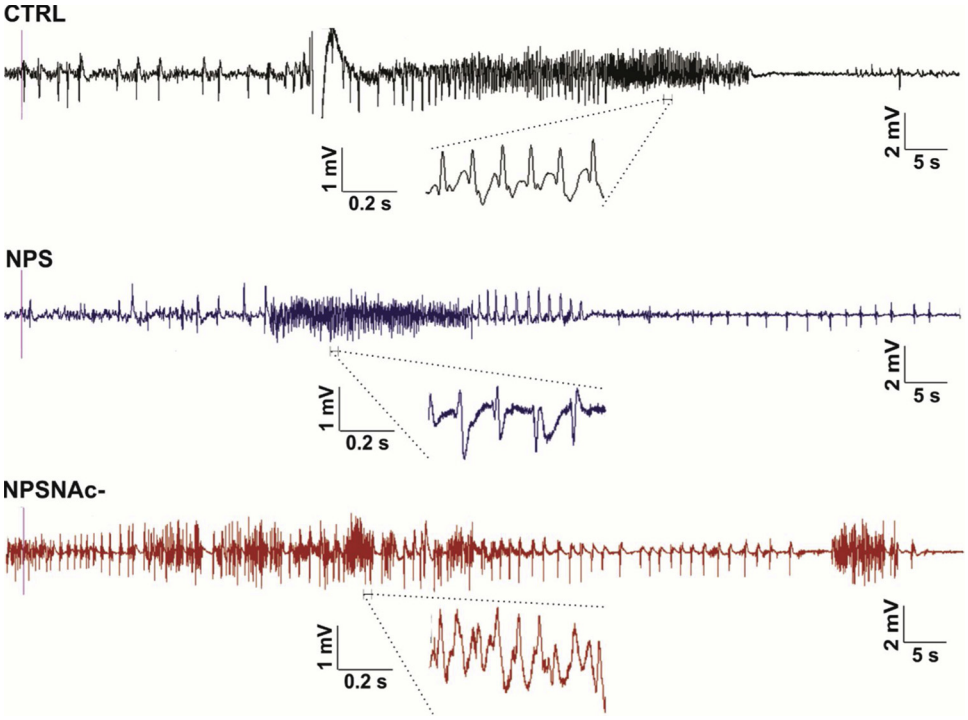

Fig. 4. Electroencephalographic recordings of epileptiform activity in the cortex of representative animals of each experimental group (top/black: CTRL; middle/blue: NPS; bottom/red: NPSNAc-). The solid vertical line in magenta, at the beginning of each tracing, indicates the onset of GTCS and simultaneous termination of PTZ infusion and of electrical stimulation. Two distinct phases (tonic and clonic) of epileptiform activity is clearly distinguishable in CTRL and NPS groups, but not in the NPSNAc- group.

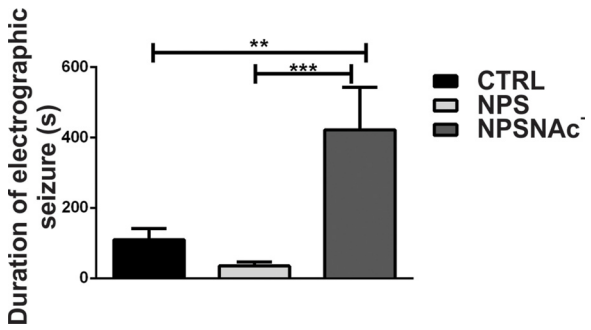

Fig. 5. Duration of epileptiform electrographic activity after the onset of GTCS. NPSNAc- displayed a longer duration of electrographic seizures when compared to both CTRL (**p < 0.01) and NPS (***p < 0.001).

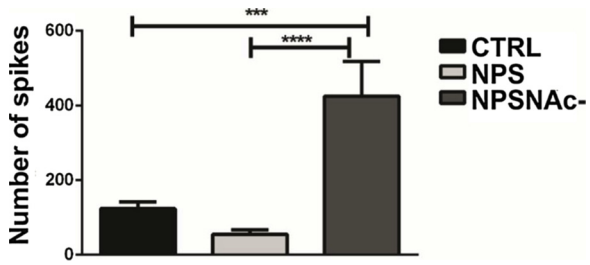

Fig. 7. Number of spikes computed across the whole duration of electrographic seizures after onset of GTCS. NPSNAc- displayed an increase in the number of spikes when compared to both CTRL (***p < 0.001) and NPS (****p < 0.0001).

NPSNAc- group. Moreover, there was also a higher count and higher firing rate of spikes in lesioned animals. Although interdependent, these three parameters reflect distinct mechanisms and their changes all point to a decrease of an inhibitory / desynchronizing tonus over ictogenic circuitry.

Epileptiform spikes are the result of the summation of excitatory post-synaptic potentials (EPSP) closer to the brain surface (Gotman, 2011; Smith, 2005). By its turn, an EPSP is the result of incoming bursts of action potentials terminating in excitatory synapses (Steriade, 1993; Niedermeyer, 2005). Thus, synchronization of neuronal activity results in shorter and higher spikes with, hence, higher spike slopes. By this token, changes in spike morphology (higher amplitude and steeper slopes) seen only in the NPSNAc- group indicate withdrawal of desynchronizing tonus over ictogenic networks during seizures induced by NAc ablation, further corroborating the idea of an important role of the structure in the suppression of seizures.

It is worth noticing that electrographic seizures of NPSNAc- animals are considerably different from those of other groups. Instead of displaying the typical evolution of the tracing with two distinct phases (tonic and clonic), epileptiform activity in this group is rather composed of multiple clusters of polyspikes with intense interictal activity between them, lasting a much longer period. Contrary to our reasoning, interictal spikes have been described as synchronous events originating from circuits unrelated to ictogenesis and that may actually have a protective role, suppressing seizures (de Curtis and Avanzini, 2001). Even though this cannot be completely ruled out, we do not believe this is the case here, given that animals from NPSNAc- group: 1) displayed the full spectrum of convulsive behavior typical of the PTZ model, culminating in generalized tonic-clonic seizures (GTCS); 2) also

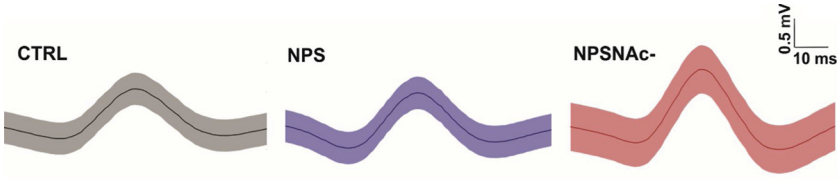

Fig. 6. Mean spike (solid line) and its standard deviation (shadow) for all epileptiform spikes across electrographic seizures from three representative animals of each experimental group (left/black: CTRL; center/blue: NPS; right/red: NPSNAc-).

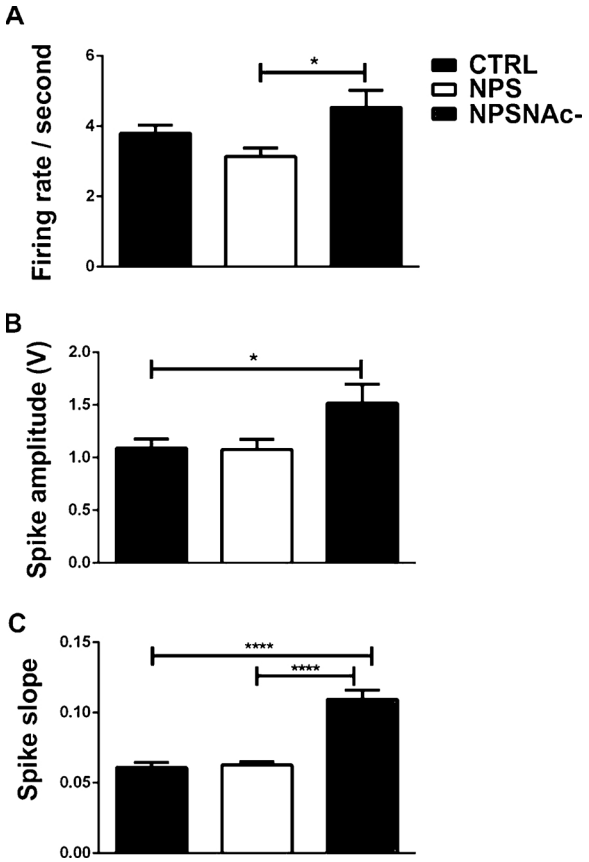

Fig. 8. Firing rate and spike morphology. NPSNAc- displayed a significant increase in firing rate when compared to NPS (*p < 0.05, one-way Anova) (panel A). Spikes from NPSNAc- animals had higher amplitudes than controls (*p < 0.05, one-way Anova) (panel B) and steeper slopes from both controls and stimulated animals (****p < 0.0001, one-way Anova) (panel C).

displayed straub tail behavior related to intense neural recruitment; 3) sustained convulsive behavior (fore and hindlimb clonus) after the termination of GTCS; 4) actually needed a smaller amount of drug to evoke GTCS (lower PTZ threshold; Fig. 3); 5) displayed recurrent epileptiform afterdischarges and; 6) died some minutes after GTCS. Moreover, the increase in interictal spiking seen here may be only a consequence of the seizure itself, such as previously observed (Goncharova et al., 2016).

Differently from previous studies of ours, NPS showed only a mild anticonvulsant effect here, indicated by the not significant trends to a shorter duration of the electrographic seizures and to a lower count of spikes (both p < 0.06), as well as by the smaller p values for statistical comparisons between NPSNAc- and NPS groups in the number of spikes and in the firing rate results. The reasons for this are unknown, but two methodological differences from previous work may have had a role: a slower PTZ infusion rate (0.4 ml/min instead of 1.0 ml/min) and the presence of recording electrodes.

It was not possible to identify a specific network for this effect of decreasing of inhibition and/or of desynchronization induced by the bilateral ablation of NAc, once cortical microsurgical screw electrodes are region unspecific, and their recordings reflect overall brain activity (Nunez and Srinivasan, 2005). On the other hand, superficial and highly synchronous firing prevails in the formation of cortical epileptiform spikes. By this token, temporal-cortical loops, with or without the participation of basal ganglia and pre-frontal areas, may have a preponderant role in present results.

In fact, connections between amygdala subnuclei with cortical and subcortical areas (including nucleus accumbens) are involved in memory processing (Roozendaal et al., 2001, 2009), attention (Maddux et al., 2007), and reward (Cardinal et al., 2003; Giertler et al., 2005; Giertler et al., 2003). These brain regions are highly interconnected (O'Doherty, 2004) and together they form an integrative network both anatomically and functionally (Goto and Grace, 2008; Russo and Nestler, 2013; Piantadosi et al., 2017). For instance, Jackson and Moghaddam (2001) showed that cortical modulation of information transfer from the amygdala to NAc is essential to translate emotional and cognitive information into an adequate motor response. Also, Ramirez et al. (2015) demonstrated that information flow from basolateral amygdala to NAc is essential to the expression of active avoidance behavior.

Present results point to the importance of NAc in epileptic phenomena. Investigation carried out by Bertram et al. (1998) suggest that epileptic seizures start in limbic structures, such as the amygdala, that recruit thalamus, which, by its turn, synchronizes epileptiform activity in many other substrates. O'Donnel et al. (1999) highlighted that NAc has dopaminergic projections to ventral pallidum which, by its turn, provides inhibitory input to the thalamocortical loop. Work done by Mahoney et al. (2018) showed that ES of the ventral pallidum is efficacious in decreasing frequency and duration of seizures in the animal model of temporal lobe epilepsy induced by pilocarpine, besides delaying PTZ-induced seizures in rats. In fact, functional connections between NAc in ventral pallidum are frequently reported (Groenewegen et al., 1993; Haber et al., 1993) and this circuit is a possible candidate to underly the effects seen in this study.

Other studies report a more direct relation of NAc with epileptic processes. Riban et al., 2004 suggested that NAc may be enrolled in the modulation of generalized epileptic seizures. Kowski et al. (2015) demonstrated that ES applied to NAc is capable of decreasing seizure frequency in patients with refractory focal epilepsy of both frontal and temporal lobe. Authors of this study advocated in favor of using ES of NAc to control seizures, even though the investigation lacks a greater number of participants. Finally, further experimental and clinical data suggest the involvement of NAc in the propagation of frontal and temporal lobe seizures, as well as behavioral modification (Schmitt et al., 2014; Long et al., 2009).

The neurochemical nature of the connection between structures in the limbic system, cortex, and NAc has also been studied. Piantadosi et al. (2017) showed that NAc receives strong glutamatergic inputs from both piriform cortex and the basolateral amygdala. Other studies pointed out that electrical stimuli delivered to the amygdala are capable of modulating neurochemical responses in the pre-frontal cortex and in the NAc (Yim and Mogenson, 1982; Jackson and Moghaddam, 2001). Of particular importance to this study, (Chergui et al., 1994) suggest that glutamatergic afferences from the basolateral amygdala and the hippocampus may exert a direct control in the increase of dopamine release in the NAc. According to Zaehle et al. (2013), the dopaminergic system of NAc has a preponderant role in the selection of inputs, being able to detect relevance (a situation in which a stimulus stand out from the background) (Cooper and Knutson, 2008). Thus, a plausible explanation for the activation of NAc by application of NPS (and not PS) to the amygdala (as seen in Mesquita et al., 2011) would be the capability of this structure of detecting salience, due to the lack of temporal structure of this ES pattern.

5. Conclusions

In summary, present behavioral and electrographic results indicate that NAc has an important role in the control of epileptiform activity induced by acute seizure models, such as PTZ infusion, and that such property is probably enhanced by application of NPS to the amygdala. This property may underly the therapeutic effect of NPS, together with direct desynchronization (Cota et al., 2016). Moreover, this mechanism of seizure suppression may be a result of indirect polysynaptic inhibition / desynchronization of ictogenic circuits that encompass the mesial temporal lobe and more widespread networks induced by the activation

of NAc which, by its turn, may interpret NPS as a salience, due to its lack of temporal structure. A more refined dissection of these highly complex circuits and effects is essential in order to better establish these ideas as facts. Authors believe that optogenetics together with opto-physiology or electrophysiology may be an interesting approach for future investigations on this topic.

6. Declaration of interest

none.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eplepsyres.2019.05.009.

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