Epilepsy & Behavior 24 (2012) 391–398

Contents lists available at SciVerse ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Behavioral and EEG effects of GABAergic manipulation of the nigro-tectal pathway in the Wistar audiogenic rat (WAR) strain II: An EEG wavelet analysis and retrograde neuronal tracer approach

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ARTICLE INFO

Article history:
Received 28 March 2012
Accepted 21 April 2012
Available online 16 June 2012

Keywords:
Audiogenic seizures
Sustained EEG oscillation
Bicuculline
Muscimol
Fluorogold

ABSTRACT

The role of the *substantia nigra pars reticulata* (SNPr) and *superior colliculus* (SC) network in rat strains susceptible to audiogenic seizures still remain underexplored in epileptology. In a previous study from our laboratory, the GABAergic drugs bicuculline (BIC) and muscimol (MUS) were microinjected into the deep layers of either the anterior SC (aSC) or the posterior SC (pSC) in animals of the Wistar audiogenic rat (WAR) strain submitted to acoustic stimulation, in which simultaneous electroencephalographic (EEG) recording of the aSC, pSC, SNPr and striatum was performed. Only MUS microinjected into the pSC blocked audiogenic seizures. In the present study, we expanded upon these previous results using the retrograde tracer Fluorogold (FG) microinjected into the aSC and pSC in conjunction with quantitative EEG analysis (wavelet transform), in the search for mechanisms associated with the susceptibility of this inbred strain to acoustic stimulation. Our hypothesis was that the WAR strain would have different connectivity between specific subareas of the superior colliculus and the SNPr when compared with resistant Wistar animals and that these connections would lead to altered behavior of this network during audiogenic seizures. Wavelet analysis showed that the only treatment with an anticonvulsant effect was MUS microinjected into the pSC region, and this treatment induced a sustained oscillation in the theta band only in the SNPr and in the pSC. These data suggest that in WAR animals, there are at least two subcortical loops and that the one involved in audiogenic seizure susceptibility appears to be the pSC-SNPr circuit. We also found that WARs presented an increase in the number of FG+ projections from the posterior SNPr to both the aSC and pSC (primarily to the pSC), with both acting as proconvulsant nuclei when compared with Wistar rats. We concluded that these two different subcortical loops within the basal ganglia are probably a consequence of the WAR genetic background.

1. Introduction

The Wistar audiogenic rat (WAR) is an inbred rodent strain susceptible to audiogenic seizures and derived from Wistar rats [1–3]. The audiogenic seizures are characterized by an initial period of wild running, jumping and atonic falling episodes followed by tonic-clonic seizures (*opisthotonus* plus forelimb and hindlimb tonic hyperextensions and clonic convulsions) ending with apnea and postictal depression [2–4].

Several studies of audiogenic strains appear in the literature, but the roles of the network formed by *substantia nigra pars reticulata* (SNPr) and *superior colliculus* (SC) still remain underexplored. The characterization of these networks is important for epileptology. When these studies are conducted in different strains, the results would show the effects of genetic variability on the circuitry affecting epilepsy. In situ hybridization studies for c-Fos mRNA showed increased labeling of the deep SC of genetically epilepsy-prone rats (GEPRs), suggesting the involvement of the deep SC in the propagation of seizures [5]. Additionally, there is a rapid burst firing of neurons in the deep SC layers in freely moving GEPRs, suggesting that this structure plays a key role in triggering the wild running behavior [6]. Strasbourg audiogenic rats have a 40% reduction of GABAergic

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doi:10.1016/j.yebeh.2012.04.133

receptors in the SNPr [7], and additional studies of GEPR animals showed that there is a lower release of GABA (stimulated by high potassium concentration) in the SNPr compared with the release observed in non-audiogenic control rats [1]. The genetically epilepsy-prone hamster (GPG/Vall) presents morphological and neurochemical abnormalities in the deep SC layers [8]. In WARs, sectioning of the unilateral or bilateral subcollicular deep SC layer significantly reduced audiogenic seizure severity. When the sectioning was performed bilaterally, the latency for running episodes, when present, was increased [9].

Neurophysiological measurements and behavioral responses have been used with success in investigating the neural networks underlying many seizure behaviors [10,11]. However, these results are dependent on the quantitative analytical methods used to extract the information from the experimental data [12]. Synchronous oscillatory activity is important in coordinating the firing of distributed neuronal populations during behavioral coding. Specific oscillatory patterns are altered in several neurological disorders, revealing abnormal network functioning [13]. The sustained aspects of these oscillations (their maintenance in time) are important, allowing us to correlate behavior with synchronization in characteristic frequency bands over time.

In our previous study [14], the GABAergic drugs bicuculline (BIC; $GABA_A$ antagonist) and muscimol (MUS; $GABA_A$ agonist) were microinjected into the deep layers of either the anterior region of SC (aSC) or the posterior part of SC (pSC) in WARs submitted to acoustic stimulation with simultaneous EEG recording of the aSC, pSC, SNPr and striatum. Only MUS microinjected in pSC blocked audiogenic seizures, following the same protocol of microinjections made in GEPRs by Merril et al. [15]. In the current study, we used quantitative analysis (wavelet transform) to study the EEG before injection (basal period), after microinjection of BIC and MUS into the deep layers of SC, during the adaptation period and during the audiogenic seizure period in WARs.

All WARs described in this study are those used in our previous paper [14]. In the current study, a new group was added and microinjected with the retrograde tracer Fluorogold (FG) to study the neuroanatomical connections comparing the SC subareas (anterior and posterior) and the SNPr between WARs and Wistar rats. In these rats, FG was microinjected into the aSC and pSC to analyze the projections from the anterior region of the SNPr (aSNPr) and posterior portion of the SNPr (pSNPr). These specific subregions of the SNPr have been respectively implicated in anticonvulsant (aSNPr) and proconvulsant (pSNPr) effects in several epilepsy models in normal rats [16–19].

Our hypothesis was that the WAR strain would have different connections between the aSC/pSC and the SNPr when compared with the resistant Wistar rat controls. If present, these differential connections would lead to different convulsant or anticonvulsant actions of this network during the expression of audiogenic seizures.

2. Methods

2.1. GABAergic drug groups

All animals and experimental procedures in these groups were the same as those used in our previous work [14]. Briefly, for additional details on the EEG experimental protocol used in Section 2.1.1 of the current study, see Rossetti et al. [14]. For the quantitative EEG evaluation (wavelet analyses) described in Section 2.1.2 of the current study, we used the same EEG recordings reported in Rossetti et al. [14].

2.1.1. Experimental procedures [14]

Fifteen male WARs (250–300 g) received phosphate buffer (PBS), BIC and MUS (Tocris; Westwoods Business Park, Ellisville, MO, USA) microinjections into the aSC (n = 6; aSC group) or pSC (n = 9; pSC group).

Prior to any manipulations, all WARs were tested for audiogenic seizures using three acoustic stimuli that were done 48 h apart (screening test). The experimental groups comprised animals that experienced a seizure with a categorized mesencephalic seizure severity index (cSI, Table 1) of at least 4 in at least one of the three screening tests. Behavioral evaluations were performed in accordance with the seizure severity index developed by Garcia-Cairasco et al. [3] and modified by Rossetti et al. [20].

Three days after the screening test, the animals were stereotaxically implanted with two twisted monopolar Teflon-coated electrodes (0.008″; AM System; Carlsborg, USA), which were placed in the striatum (anterior–posterior (AP) = 3.0 mm; lateral (L) = +2.3 mm; ventral (V) = −5.0 mm) and SNPr (AP = −5.4 mm; L = +2.3 mm; V = −8.0 mm). One chemitrode, an electrode coupled with a cannula (for simultaneous recording and drug microinjections), was implanted in the aSC (AP = −6.4 mm; L = +1.5 mm; V = −5.0 mm) or pSC (AP = −7.3 mm; L = +1.5 mm; V = −4.47 mm; $\hat{A} = 28°$ refers to the sagittal plane). All coordinates were obtained from the atlas of Paxinos and Watson [21] and are relative to the bregma suture.

Seven days after the surgery, the animals were unilaterally microinjected with 200 nl of PBS (50 mM, pH = 7.4), stimulated with sound and video-EEG recorded. After 48 h, the animals were unilaterally microinjected with 200 nl of BIC (0.1 nmol, according to Merrill et al. [15]) and then stimulated with sound and video-EEG recorded. The animals were unilaterally microinjected with 200 nl of PBS and then stimulated with sound and video-EEG recorded 48 h later. After an additional 48-h period, the animals were unilaterally microinjected with 200 nl of MUS (1.75 nmol, according to Rossetti et al. [20]) and then stimulated with sound and video-EEG recorded. All the EEG recording procedures were conducted according to Dutra Moraes et al. [22] and are detailed in Rossetti et al. [14].

At the end of the experiments, the animals were perfused with 70 ml of PBS (50 mM, pH 7.4) followed by 300 ml of paraformaldehyde/PBS (4%, pH 7.4). The brains were removed and post-fixed in 4% paraformaldehyde for 1 h, cryoprotected in 20% sucrose for 24 h at 4 °C, frozen in isopentane and sliced into 40-μm-thick coronal sections on a cryostat (Micron-Zeiss HM-505-E; Walldorf, Germany). The sections were mounted on gelatin-subbed glass slides and stored at −20 °C until Nissl staining was used to reveal the correct electrode and chemitrode placement.

2.1.2. Quantitative EEG evaluation

To evaluate the effects of drugs tested in WARs, a time–frequency spectral analysis using wavelet transform [12,20] was performed on the EEG traces. EEG tracing is a method developed for the analysis of non-stationary signals by separating the EEG time-series into its temporal and spectral components. This method has already been used in several studies of the correlation of EEG oscillatory patterns and behavioral responses [23–25].

We analyzed frequencies between 3 and 20 Hz during the basal period (6 min prior to any manipulation), adaptation period (6 min prior to acoustic stimulation) and audiogenic seizure period.

Table 1
SI with behavioral descriptions according to Garcia-Cairasco et al. [3], categorized into discreet variables for statistical purposes by Rossetti et al. (cSI; [20]).

SI	Seizure behaviors	cSI
0.00	No seizures	0
0.11	One running	1
0.23	One wild running (running plus jumping plus atonic falling) (WR1)	2
0.38	Two wild runnings (WR2)	3
0.61	Tonic convulsion (*opisthotonus*)	4
0.85	Tonic seizures plus generalized clonic convulsions (TS)	5
0.90	Head ventral flexion plus cSI 5	6
0.95	Forelimb hyperextension plus cSI 6[a] (FH)	7
1.00	Hindlimb hyperextension plus cSI 7[a] (HH)	8

[a] Categories that are generally followed by hindlimb clonic convulsions (HCC).

For basal and adaptation periods, 5 to 6 min of EEG recording were divided in 10-s epochs. The entire recording period was analyzed at 30 epochs per rat. In these epochs, we calculated the value of the most prominent frequencies in Hz (F1; e.g., the frequency with the highest power (in μV^2/Hz) calculated using the MATLAB program) and the duration to characterize sustained oscillations. We defined sustained oscillations as all frequency oscillations with durations of at least 30% of the epoch.

During the audiogenic seizure period, the EEG was recorded (the amount of time varied between rats but usually lasted approximately 30 s per seizure) and divided into epochs according to the duration of seizure behavior types, as determined by the audiogenic severity index (SI; Rossetti et al. [20]; Table 1): wild running 1 (cSI = 2), 3 s; wild running 2 (cSI = 3), 9 s; tonic seizures (cSI = 4), 1.5 s; period between wild running 1 and 2, 10 s.

2.2. FG groups

Twelve naïve male WARs and twelve naïve male Wistar rats (250–300 g) were injected with FG (2% in PBS; Fluorochrome, LLC; Denver, Colorado, USA) into the aSC (6 WARs and 6 Wistar rats) or pSC (6 WARs and 6 Wistar rats). These animals were used for anatomical analyses.

Water and food were available ad libitum to the rats. Room conditions were controlled within a 23 °C to 25 °C range and a 12-h light/dark cycle. All experiments were performed in accordance with the recommendations of the Brazilian Society for Neuroscience and Behavior for animal experimentation. Every effort was made to avoid unnecessary animal suffering and to reduce the number of animals used. All experimental procedures used in this study are in accordance with the Ethical Commission of Ethics in Animal Research at the Ribeirão Preto School of Medicine at the University of São Paulo (protocol number 072/2007).

2.2.1. Stereotaxic surgery and microinjection of FG

All of the naïve WARs and Wistar animals were anesthetized with tribromo-ethanol (2.5%, 1 ml/100 g of body weight; Sigma-Aldrich; Milwaukee, USA) and supplementary doses of ketamine (10%; 0.1 ml/100 g of rat weight; Agener União; SP, Brazil) with xylazine (2%, 0.007 ml/100 g of rat weight; Rompun, Bayer; SP, Brazil) diluted in saline 0.9%, given when needed. Then, the animals were implanted with cannulas into the deep layers of the aSC (AP = −6.4 mm; L = +1.5 mm; V = −5.0 mm) or pSC (AP = −7.3 mm; L = +1.5 mm; V = −4.47 mm; Â = 28° refers to the sagittal plane). All coordinates were obtained from the atlas of Paxinos and Watson [21] and are given relative to the bregma suture. Immediately after cannula implantation, with the animal anesthetized in the stereotaxic apparatus, 50 nl of FG was microinjected into the aSC or pSC. After 10 min, the animal's head was sterilized with saline 0.9% and oxygen peroxide and then sutured.

2.2.2. Histology

After 7 days, the animals were anesthetized with an overdose (0.5 ml) of thionembutal (40 mg/ml; Abbott Laboratories; North Chicago, USA) and perfused with 70 ml of PBS followed by 300 ml of paraformaldehyde/PBS 4%. The brains were removed and post-fixed in paraformaldehyde 4% for 1 h. Then, the brains were cryoprotected in 20% sucrose for 24 h at 4 °C before being frozen in isopentane and sliced into 40-μm-thick coronal sections on a cryostat (Micron-Zeiss HM-505-E; Walldorf, Germany) as follows:

1− From AP = −4.5 mm to AP = −5.5 mm of the ipsilateral SNPr, relative to the bregma [21]; 6 coronal sections (40 μm) were cut 120 μm apart. This SNPr area, in agreement with the literature [17,26,27], was designated as the aSNPr.
2− From AP = −5.51 mm to AP = −6.5 mm of the ipsilateral SNPr, relative to the bregma [21]; 6 coronal sections (40 μm) were cut 120 μm apart. This SNPr area, in agreement with the literature [17,26,27], was designated as the pSNPr.

The 120-μm distance between sections assured that during histological analyses, we did not count the same neuron twice. Additionally, we performed immunofluorescence staining for the $GABA_A$ receptor β2.3 subunit (1:1000; Upstate Technology; New York, USA) in all sections to highlight the ipsilateral SNPr limits and to facilitate Fluorogold-positive cell counts. These sections underwent immunohistochemistry according to the following protocols: 1st day — the sections received 5 baths of PBS for 5 min (0.1 M, pH = 7.4). Afterward, the sections were incubated in 0.1 M glycine for 5 min, and again received 2 baths of PBS for 5 min and were immediately incubated in 1% BSA for 30 min. Then, the sections were incubated overnight with the primary antibody for the $GABA_A$ receptor β2.3 subunit (1:1000). In the control sections, this final step was not performed. 2nd day — the sections received 5 baths of PBS for 5 min and immediately incubated with an Alexa 488-conjugated secondary antibody (1:2000) for 1 h and 20 min, protected from light. Then, the sections received 6 baths of PBS for 5 min. All sections were placed on slides that were mounted with Fluoromount-G (Southern Biotech, Birminghan, USA).

2.2.3. Image acquisition of sections and counting of SNPr neurons

Image acquisition was performed using Image-Pro Plus (Media Cybernetics, Inc.; Silver Spring, USA) software installed on a personal computer coupled to a motorized Olympus BX 60 microscope. Microscopic fields were of mosaic compositions made using the enhanced depth field (EDF) techniques of the Image-Pro Plus (Media Cybernetics, Inc.; Silver Spring, USA). Using these techniques, we were able to

Table 2
Average ± S.E.M of the most prominent frequency (F1) and its sustained oscillation (SO) on the basal period in all of the analyzed structures. **p<0.01; ***p<0.001; one-way Kruskal–Wallis test with Dunn's post-hoc analysis.

	Striatum	SNPr	aSC	pSC
F1 (Hz)	4.25 ± 0.07	4.65 ± 0.10	4.13 ± 0.10	5.06 ± 0.09***
SO (s)	4.17 ± 0.11	4.47 ± 0.14	5.10 ± 0.19	5.48 ± 0.22**

Table 3
Average ± S.E.M of the most prominent frequency (F1) and its sustained oscillation (SO) in all of the analyzed structures of WARs that received injections in the aSC. *p<0.05; one-way Kruskal–Wallis test with Dunn's post-hoc analysis showing the differences between GABAergic drug *treatment* oscillation and *basal* oscillation (Table 2) within the same structure.

		Striatum	SNPr	aSC (injected site)
Bicuculline	F1 (Hz)	4.86 ± 0.10*	4.16 ± 0.13	4.07 ± 0.10
	SO (s)	4.44 ± 0.14	4.70 ± 0.22	5.04 ± 0.21
Muscimol	F1 (Hz)	4.16 ± 0.11	4.41 ± 0.17	4.44 ± 0.10
	SO (s)	4.68 ± 0.22	4.56 ± 0.23	4.79 ± 0.23

Table 4
Average ± S.E.M of the most prominent frequency (F1) and its sustained oscillation (SO) in all of the analyzed structures of WARs that received injections in the pSC. *p<0.05; ***p<0.001; one-way Kruskal–Wallis test with Dunn's post-hoc analysis showing the differences between GABAergic drug *treatment* oscillation and *basal* oscillation within the same structure.

		Striatum	SNPr	pSC (injected site)
Bicuculline	F1 (Hz)	4.29 ± 0.11	4.24 ± 0.15	4.41 ± 0.10***
	SO (s)	4.42 ± 0.15	4.45 ± 0.28	4.73 ± 0.18*
Muscimol	F1 (Hz)	4.07 ± 0.08	4.65 ± 0.15	4.39 ± 0.10***
	SO (s)	4.77 ± 0.16	4.72 ± 0.25	4.89 ± 0.15*

count the neurons in detail because each piece of the mosaic in the panoramic view could be observed with high magnitude (ex: 10×) and good resolution (see Fig. 3A). Moreover, we were able to conduct three-dimensional cell counting through the entire slice.

We used the mosaic images to count FG-positive neurons in the anterior and posterior SNPr into the nigral network. This work was performed with the aid of the manual tag technique (Image Pro-Plus) and Adobe Photoshop CS3 (Adobe System Incorporated, USA).

2.3. Statistical analyses

For both experimental groups (GABAergic drugs and FG), statistical analyses were performed using the SigmaStat 3.1 software (Systat Software UK Limited; London, UK). All data were initially tested for normality and followed by the one-way ANOVA test with Tukey's and Dunn's post-hoc comparisons. The differences were considered significant for $p<0.05$.

3. Results

3.1. Wavelet analyses of GABAergic drug groups

3.1.1. Basal period

During the basal period, the nuclei oscillate normally in the theta band (4 to 8 Hz), but the pSC oscillated with the highest frequency and duration, oscillating for 50% more than the average time (Table 2).

3.1.2. Adaptation period

The injections of PBS into both nuclei (aSC and pSC) caused no changes in the F1 or SO in any of the studied structures (data not shown). The microinjection of BIC into the aSC caused an increase in the F1 oscillation of the striatum (Table 3). The aSC nucleus did not present any changes in its oscillation. Additionally, the microinjection of MUS into the aSC caused no effects on the oscillation of either the striatum or the SNPr. However, microinjections of

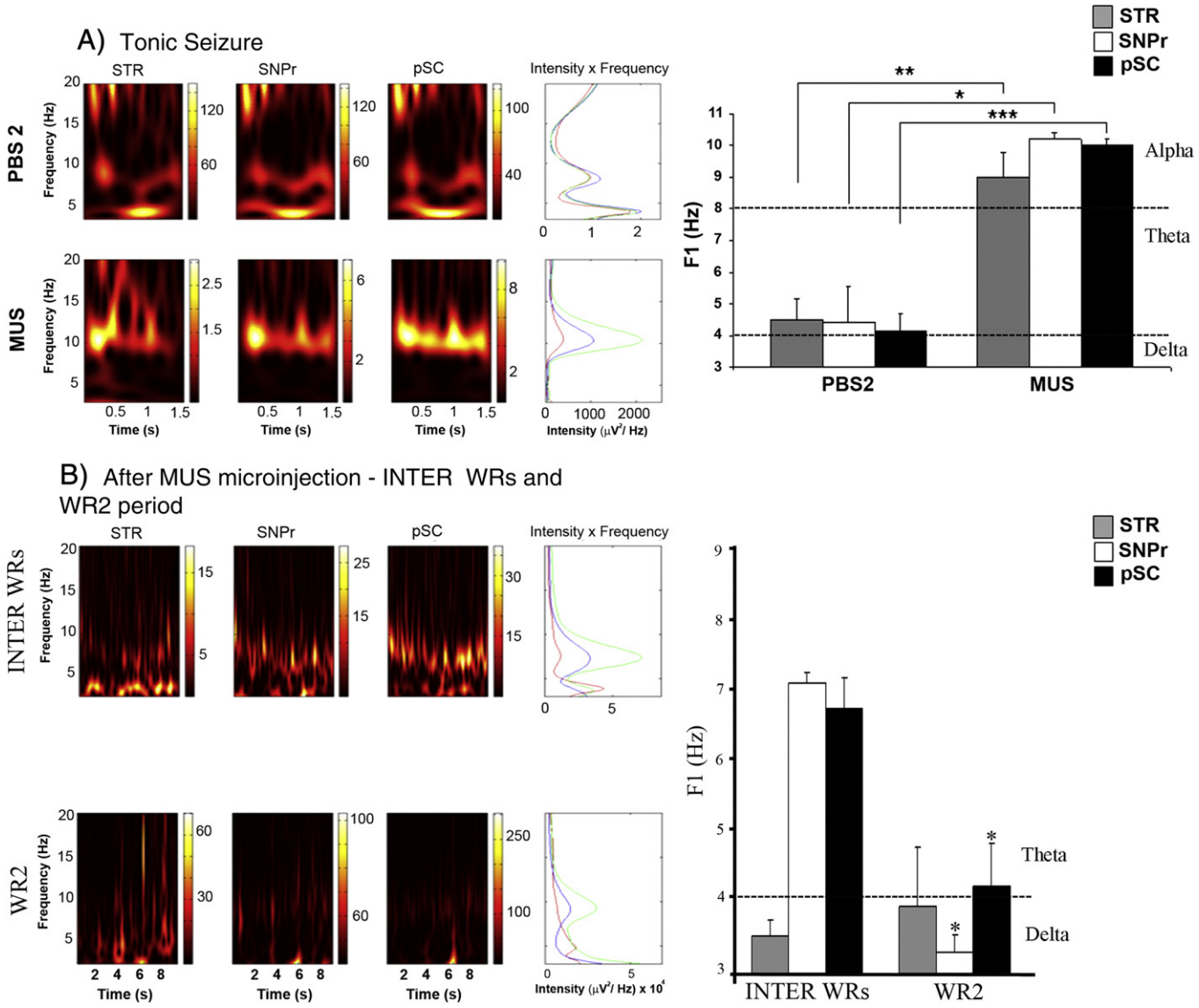

Fig. 1. Representative graphics of the wavelet transform analyses and the first most prominent frequency (F1) graphic of A — tonic seizure (TS) comparing PBS and MUS microinjections; B — interval between the first and second wild running called INTER WRs compared with the second wild running (WR2). All the behaviors and periods analyzed in this graphic correspond to the pSC group. After performing mathematical transformations, the component frequencies (in Hz) of the trace emerge, and their intensities (μV^2/Hz) are plotted in the frequency × intensity graphs and represented by the following: red line (STR), blue line (SNPr) and green line (pSC). In the time × frequency graphs, the power of the spectrum is represented as calibration bars within each graph. ***$p<0.001$, **$p<0.01$, *$p<0.05$, Kruskal–Wallis test with Tukey's post-hoc test. Hot colors correspond to greater intensity values in each frequency. Abbreviations: PBS2 — second PBS microinjection; MUS — muscimol microinjection; STR = striatum; SNPr = substantia nigra pars reticulata; pSC = posterior superior colliculus.

GABAergic drugs (MUS and BIC) into the pSC reduced both F1 and SO (Table 4).

3.1.3. Audiogenic seizure period

The decrease of F1 and SO in the pSC caused by MUS microinjection coincided with the anticonvulsant behavioral effects during the sound stimulation. 7 of the 9 WARs had partially blocked seizures during the tonic phase (*opisthotonus*; cSI = 4) and with complete absence of clonic seizures and tonic hyperextension behaviors. Two of the nine WARs that were microinjected did not present any behavioral seizures. Early termination of seizures correlated with increased F1 and SO during tonic seizures in all structures, primarily the SNPr and pSC, compared with animals that presented complete audiogenic seizures. In the striatum, SNPr and pSC, there was an increase of F1 in the alpha band (8 to 12 Hz) but there was only a significant difference of SO between the SNPr and the pSC (Fig. 1A).

Additionally, following the first wild running, there was a period that we called INTER-WRs, which was characterized by the absence of seizures followed by a second wild running period. During this second wild running period, F1 increased in the SNPr and pSC in a similar manner until the early end of audiogenic seizure period. However, in this period, F1 oscillated in the theta band (between 6 and 8 Hz; Fig. 1B). These effects did not occur after BIC microinjections (data not shown).

3.2. FG groups

Only animals with accurate microinjections of FG in the deep layers of aSC and pSC were considered for analyses: aSC group (WARs: n = 4; Wistar rats: n = 4); pSC group (WARs: n = 4; Wistar rats: n = 5) (Fig. 2).

$GABA_A$ receptor immunofluorescence allowed us to delineate the SNPr because this specific nucleus is rich in $GABA_A$ receptors. Only FG+ neurons of the anterior SNPr and posterior SNPr in this region were counted (Fig. 3).

The quantitative analysis of FG+ cells showed that the number of neurons projecting from the posterior SNPr to both the aSC and pSC regions was higher in WARs than in Wistar rats (Fig. 4) and that the number of projections from the posterior SNPr to both regions of the SC was higher than the projections from the anterior SNPr.

4. Discussion

BIC microinjection into the aSC increased F1 in the striatum, and GABAergic (MUS and BIC) microinjections into the pSC decreased F1 and SO during the adaptation period. Our results clearly demonstrate that the SC in WARs has two defined regions that express distinct EEG features and receptor phenotypes (or chemical features) as observed with different responses to GABAergic drugs. This finding corroborates our preliminary division of SC into two regions, which we initially formulated due to the literature descriptions of three different subcortical loops that are formed by connections between the basal ganglia nuclei, SC and thalamus [28] and characterized in our previous studies [14]. The SC receives projections from the SNPr in three different regions (one from the superficial layers and two from the deep layers) that make connections with the thalamus. The latter sends projections into the striatum, which also sends projections back into the SNPr, closing the loop [28]. However, none of the

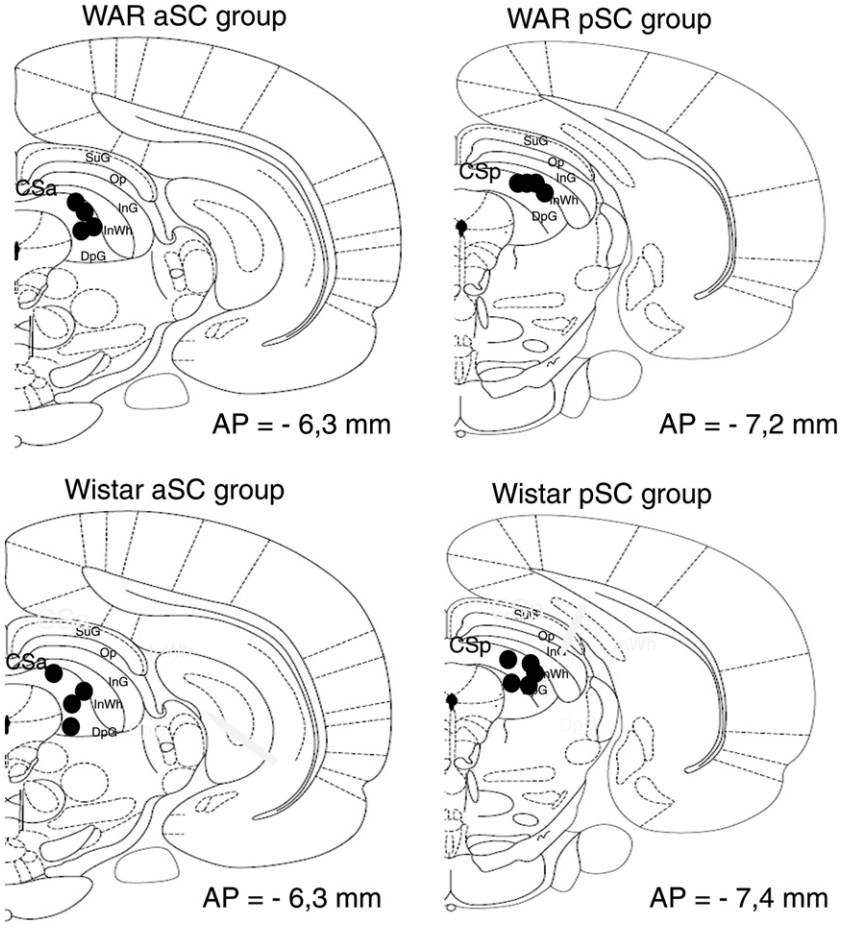

Fig. 2. Microinjection locations of FG into the aSC and pSC. All coordinates were obtained from the atlas of Paxinos and Watson [21] (AP — antero-posterior in mm, relative to the bregma suture). WAR aSC group (n = 4); WAR pSC group (n = 4); Wistar aSC group (n = 4) and Wistar pSC group (n = 5).

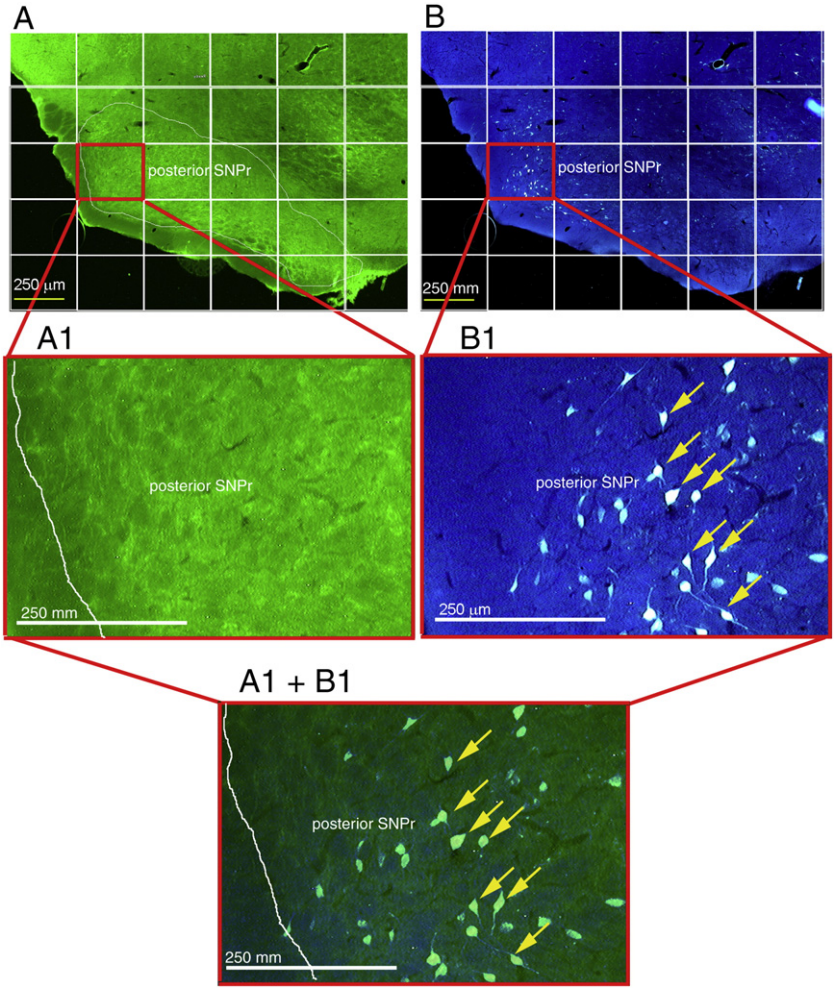

Fig. 3. GABA-FG co-localization in the posterior SNPr. A — Mosaic reconstruction of immunofluorescence for $GABA_A$ receptor subunit β2.3 (green). Drawn lines indicate the limits of the posterior SNPr. Only the neurons that were located within these limits were used for GABA-FG co-localization counts. B — Mosaic reconstruction of FG retrograde-labeled neurons in the posterior SNPr (cyan). Mosaic matrixes were composed of 30 acquisitions; 5 along the y-axis and 6 along the x-axis. A1 and B1 — Posterior SNPr mosaic with 10× zoom. A1 + B1 — Posterior SNPr mosaic representing the co-localization between $GABA_A$ receptors subunit β2.3 (in green) and FG+ neurons (in cyan). Yellow arrows: cellular bodies of posterior SNPr neurons that send projections into pSC.

microinjections into the aSC produced an anticonvulsant effect during sound stimulation, unlike MUS, which when microinjected into pSC reduced seizures during the stimuli. Therefore, the aSC and pSC possibly form two subcortical loops connected with the basal ganglia and thalamus, with the pSC acting as a proconvulsant nucleus directly involved in the activation (or disinhibition) of the audiogenic seizure circuitry in WARs.

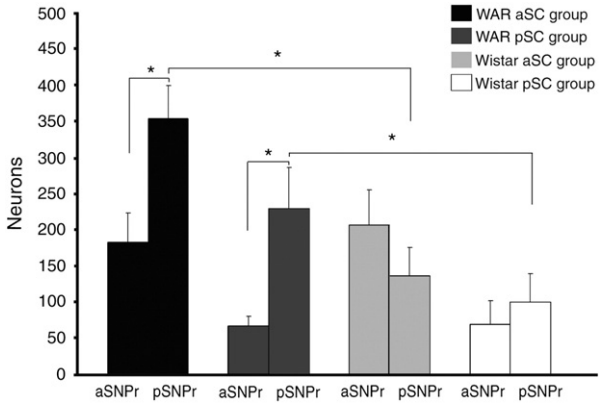

Fig. 4. Mean ± S.E.M of numbers of FG+ neurons in the anterior SNPr (aSNPr) and posterior SNPr (pSNPr). Groups: WARs microinjected in the anterior SC (WAR aSC group, n = 4) and posterior SC (WAR pSC group, n = 4); Wistar rats that were microinjected in the anterior SC (Wistar aSC group; n = 4) and posterior SC (Wistar pSC group; n = 5). *$p < 0.05$, Kruskal–Wallis test with Tukey's post-hoc test.

Our study also shed light onto the joint action of the SNPr and pSC. Increased F1 of these nuclei occurred in two crucial moments during audiogenic seizures of the animals that received MUS microinjections. The first of these was during a period we called INTER-WRs that appeared only in these animals, between the first wild running and second wild running. This period was characterized by sustained high frequencies oscillation of the EEG (theta band) of the SNPr and pSC, along with no exhibition of behavioral seizures. The second crucial moment was during the final period of the audiogenic seizures. In this case, during tonic seizures, the F1 (alpha band) increased simultaneously only in the SNPr and pSC. We hypothesized that for the first period, these nuclei were activated as a protective mechanism, stopping the wild running in a functional attempt to stabilize the system and terminate the seizures. In fact, if we stop the sound stimulus at this stage, the seizures would also stop. In our experience, this happens normally when we stop the sound during the first wild running: audiogenic seizures are automatically stopped, but, because the sound continues, the animals start a second wild running. Therefore, when a WAR enters its second wild running episode, seizures follow their course until the end, with or without sound stimulation, as far

as tonic seizures occur (the presence of tonic seizure is the criterion for stopping the sound). We concluded that in this exact moment of the audiogenic seizure, after the first running and before the second, both the SNPr and pSC act in conjunction to stop the seizures with higher frequency than the one presented in the INTER-WRs period. This result is even more evident in another study from our group that used phenobarbital (PHE) systemic injection; the EEG oscillation of the SNPr and SC decreased both during the adaptation period and during the sound stimulation in animals that had audiogenic seizures blocked by PHE [20]. When the animal had seizures, there was an increase of F1 in both nuclei, which returned to normal when the seizures ended [20]. Nevertheless, when we bilaterally injected either PHE or MUS into the SNPr, the audiogenic seizures of WARs were not blocked and temporally coincided with an increase of F1 in the SC. The animals that had audiogenic seizures presented simultaneous oscillations (alpha band) of the SNPr and SC during hindlimb clonic seizures, cSI = 8 [20].

The participation of the SNPr in seizure activity has been discussed in the literature. Microinjections of dopamine into the anterior SNPr in Wistar rats induced preconvulsive behaviors, such as staring, immobilization, facial and mouth movements and wet dog shakes associated with Fos oncoprotein expression in the limbic system [27] and in the RNAm expression of the glutamatergic receptors GLUR1, GLUR2 and NMDAR1 [17]. Microinjection of dopamine into the posterior SNPr in Wistar rats showed neither pre-convulsive behavior nor Fos expression [27]. According to these authors, the specific effects in the anterior SNPr can be due to the action of dopamine in D1 receptors, which are located in GABAergic neuronal terminals that cause GABA release and therefore provoke reduction of their activity and, consequently, cause disinhibitory effects in the thalamic nuclei. Therefore, when the anterior SNPr of Wistar rats is inhibited, preconvulsive behaviors appear. This does not happen when the posterior SNPr is inhibited [16,17,27]. The authors conclude that in the Wistar strain, the anterior SNPr acts as an anticonvulsant nucleus, whereas the posterior SNPr acts as a proconvulsant nucleus.

In our FG studies, we observed that the neuronal projections from the anterior and posterior SNPr to both aSC and pSC regions form different connectivity patterns in WARs compared to those from Wistar animals. In WARs, the posterior SNPr sends more projections to both SC regions than the anterior SNPr. The genetic background of the WARs might explain the differences in the connectivity between the SNPr and SC, mainly in the posterior SNPr, which is probably crucial to their audiogenic seizure susceptibility. This finding is interesting because according to Fan et al. [16,17,27], the posterior SNPr is called the proconvulsant region of SNPr in Wistar animals. Because WAR is a Wistar-derived strain, the proconvulsant feature of the posterior SNPr is preserved in WARs; it is tempting to speculate that the higher number of projections from the proconvulsant region of the SNPr to the pSC would be an important functional and neuroanatomical component to explain the audiogenic susceptibility of the WARs.

In an animal seizure model induced by fluorothyl in Sprague–Dawley rats, bilateral microinjections of (Z)-3-[(aminoiminomethyl) thio]prop-2-enoic acid (ZAPA, an agonist of the low-affinity $GABA_A$ receptor site), gamma-vinyl-GABA and MUS into the anterior SNPr caused anticonvulsant effects. However, the same microinjections into the posterior SNPr caused proconvulsant effects [19,29]. The microinjections of BIC (100 ng) were proconvulsant in the anterior SNPr but ineffective in the posterior SNPr [19]. These studies characterized the anterior SNPr as a proconvulsant nucleus and the posterior SNPr as an anticonvulsant nucleus in the Sprague–Dawley strain, which can be compared with our findings with WARs, a genetically developed strain.

One final comment can be made on the impact of the current study to the proposal of SNPr as a potential target for deep brain stimulation. Conflicts in the literature are mostly due to the use of normal rats made epileptic by different, usually chemical, treatments [30,31], in contrast with the endogenous epileptogenicity of WARs.

In conclusion, the SC in WARs presents two functional sub-regions defined with distinct GABAergic drug response and EEG characteristics, forming at least two subcortical loops. The genetic background of the WARs certainly causes important changes in the neuronal projections between the posterior SNPr and the aSC and pSC regions, compared with the WAR parent strain (Wistar). This new pool of data strongly supports the view that changes in the nigro-tectal pathway might be one of the causes of audiogenic seizure susceptibility condition. In addition, only the pSC participates in the audiogenic seizure circuitry in WARs as a proconvulsant nucleus, possibly due to the increased number of projections from the posterior SNPr, a nucleus also found to be proconvulsant in the Wistar strain when challenged with GABAergic drugs [16,17,27].

Acknowledgments

Franco Rossetti, Marcelo C.A. Rodrigues, Simone S. Marroni, Artur Fernandes and Maira Lícia Foresti were supported by FAPESP fellowships (03/12420-2, 03/01134-9, 05/58939-4, 05/513366-9, 06/60768-6, respectively). Rodrigo Neves Romcy-Pereira was supported by a PRODOC-CAPES fellowship, and Dráulio Barros de Araújo was supported by FAPESP, CNPq and CAPES. Norberto Garcia-Cairasco holds a CNPQ Research Fellowship and is supported by FAPESP (2003/00873-2;2007/50261-4), FAPESP-Cinapce (2005/56447-7), CNPq, PRONEX, CAPES-PROAP-PROEX and FAEPA grants. The present study was also supported by FAPESP (03/11381-3, 03/00873-2) grants. Thanks to Flávio Del Vecchio and José Antonio Cortes de Oliveira for technical assistance and to Leonardo Fidelis Filho and Eduardo Gomes for the care of the animals at the *vivarium* of the Physiology Department of the Ribeirão Preto School of Medicine at the University of São Paulo, São Paulo, Brazil. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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