HHS Public Access

Author manuscript

Epilepsia. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

Epilepsia. 2015 November ; 56(11): 1793–1802. doi:10.1111/epi.13204.

The role of the substantia nigra pars reticulata in kindling resistance in rats with genetic absence epilepsy

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Summary

Objective—Genetic Absence Epilepsy Rats from Strasbourg (GAERS) show a resistance to secondary generalization of focal limbic seizures evoked by kindling. The substantia nigra pars reticulata (SNR) is involved in the propagation and modulation of seizures in kindling. We first examined the role of the $SNR_{anterior}$ and $SNR_{posterior}$ subregions in the resistance to the development of kindling in GAERS. Subsequently, to determine whether kindling resistance relates to differential sensitivity of GABAergic or dopaminergic SNR neurons to kindling, we studied the effects of kindling-inducing stimulations on parvalbumin (PRV; GABAergic neuron marker) or tyrosine hydroxylase (TH; dopaminergic neuron marker) immunoreactivity (ir) respectively in GAERS and in nonepileptic control (NEC) Wistar rats that lack kindling resistance.

Methods—Adult male GAERS were implanted with a stimulation electrode in the amygdala and bilateral injection cannulas for lidocaine or saline injection (30 min before each kindling stimulation until the animals reached three stage 5 seizures or the 22 stimulations) into the

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Disclosure: No author has any disclosures relating to data published in this manuscript. 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.

$SNR_{anterior}$ or $SNR_{posterior}$. In another experiment, PRV-ir in $SNR_{anterior}$ and $SNR_{posterior}$ and TH-ir in $SNR_{posterior}$ only were densitometrically compared in GAERS-SHAM, NEC-SHAM GAERS-STIM and NEC-STIM animals (6 kindling stimulations).

Results—Bilateral $SNR_{posterior}$ infusions of lidocaine eliminated the kindling resistance and resulted in stage 5 generalized motor seizures in all kindled rats. Bilateral lidocaine infusions in the $SNR_{anterior}$ failed to alter the kindling resistance in GAERS. PRV-ir in the $SNR_{posterior}$ was unaltered in GAERS-STIM but increased in NEC-STIM group. Cellular TH-ir in the $SNR_{posterior}$ significantly increased by kindling stimulations in both NEC-STIM and GAERS-STIM groups.

Significance—The kindling resistance in GAERS is mediated by the $SNR_{posterior}$ in a lidocaine-sensitive manner. The insensitivity to kindling stimulation of PRV-ir in $SNR_{posterior}$ of GAERS but not NEC rats, implicate GABAergic $SNR_{posterior}$ neurons in kindling resistance. In contrast, the observed stimulation-specific increase in TH-ir in the $SNR_{posterior}$ is unrelated to kindling resistance.

Keywords

GAERS; Kindling resistance; Substantia nigra pars reticulata; Tyrosine hydroxylase; Parvalbumin

The observation that the coexistence of typical absence epilepsy and mesial temporal lobe epilepsy in the same patient is extremely rare has raised a question about the interactions of absence epilepsy and mesial temporal lobe epilepsy.[1,2] This question has been examined in two well-validated genetic models of absence epilepsy, Genetic Absence Epilepsy Rats from Strasbourg (GAERS) and Wistar Albino Glaxo Rats from Rijswijk (WAG/Rij) rats. The WAG/Rij and GAERS rats show a resistance to, or a delay of, secondary generalization of focal limbic seizures evoked by kindling, which here serves as a model of temporal lobe epilepsy.[3-7] In accordance with these findings, electrical co-stimulation of the thalamic reticular nucleus during hippocampal kindling suppresses the development of generalized limbic seizures in adult Wistar rats.[8] Further, a digenic mouse model, expressing two epilepsy-associated ion channel mutations that are pathogenic for absence epilepsy and temporal lobe epilepsy, reveals a protective interaction ameliorating epilepsy phenotype.[9] These findings suggest an interaction between the limbic circuits stimulated by kindling and the cortico-thalamo-cortical networks involved in absence epilepsy.

The substantia nigra pars reticulata (SNR) plays central role in controlling the activity of both corticothalamic and limbic networks.[10,11] The SNR neurons, which are involved in the cognition and coordination of motor functions[12], have GABAergic inputs from striatum and external globus pallidus and glutamatergic inputs from subthalamic nucleus.[13] The major output targets of the SNR include thalamus, midbrain reticular formation and superior colliculus.[14] The SNR consists of primarily GABAergic neurons, the majority of which are labeled by parvalbumin (PRV).[15] A small population of tyrosine hydroxylase (TH) positive dopaminergic neurons is present at the posterior SNR ($SNR_{posterior}$).[15,16]

Several lines of evidence indicate that the SNR is involved in the modulation, propagation and cessation of different types of experimental seizures including kindling or absence epilepsy.[11,17-20] There is evidence that there are two distinct subregions in the adult male

SNR, $SNR_{anterior}$ and $SNR_{posterior}$ with different roles in the propagation and control of epileptic seizures.[11,21,22] Pharmacological studies showed that bilateral microinfusions of the $GABA_A$ receptor agonist, muscimol, into the $SNR_{anterior}$ produce anticonvulsant effects whereas muscimol infusions into the $SNR_{posterior}$ have proconvulsant effects in adult male rats.[23] In contrast, microinfusions of the $GABA_A$ receptor antagonist, bicuculline, were proconvulsant into the $SNR_{anterior}$ and without any effect in the $SNR_{posterior}$.[24]

In the present study, we tested the role of $SNR_{anterior}$ or $SNR_{posterior}$ regions in the resistance to the development of generalized seizures during kindling in adult GAERS. In the first group of experiments, we suppressed the activity of the SNR during the development of kindling using localized infusions of lidocaine, previously shown to produce a reversible deactivation of neuronal activity.[5] In a second experiment we assessed whether there are differences in the two subregions of the SNR in the response of GABAergic and dopaminergic SNR neurons to kindling stimulation. We specifically compared the effects of six kindling stimulations on PRV or TH immunoreactivity (ir) while keeping all NECs and GAERS at seizure stage 2.

Methods

Animals and experimental design

Adult male Wistar non-epileptic control (NEC) (n=10), and GAERS (n=42) 4–6 month-old, rats (250-350 g) were used. The animals were maintained under standard laboratory conditions on a 12/12-h light/dark cycle and were allowed free access to food and water. All experiments were done with the approval of the Marmara University Ethical Committee for Experimental Animals (02.2011.mar) were planned in accordance with the ARRIVE Guidelines and Basel Declaration. Rats were assigned to the experimental groups as defined in Supporting Information Figure 1.

Stereotaxic surgery

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Stimulation and recording electrodes (Plastics One, VA, USA) were inserted for all animals in the right basolateral amygdala (BLA) (AP-2.6 mm, ML+4.8 mm, V-8.5 mm) and three single-contact electrodes were placed over the left cortex (frontal cortex: AP +2.0 mm, ML-3.5mm; occipital cortex: AP −6.0 mm, ML-4.0 mm; ground electrode on the cerebellum). In addition, in the first experiment, injection guide cannulas were implanted bilaterally into the $SNR_{anterior}$ or $SNR_{posterior}$ (AP-5.2 mm, ML±4.4 mm, V-7.2 mm for $SNR_{anterior}$ and AP-5.8 mm, ML±4.6 mm, V-7.4 mm for $SNR_{posterior}$) with 15° angle for the subsequent injections All coordinates were obtained from the stereotaxic atlas of Paxinos and Watson.[25] Following surgery, the rats were housed singly in Plexiglas cages.

Kindling

Kindling experiments were done as previously reported.[4] One week after the stereotaxic surgery, after discharge (AD) thresholds were determined for animals destined to undergo kindling. After the AD threshold determination, stimuli were delivered at the current AD threshold twice daily in the morning and late afternoon (9.30 am-4.30 pm). In the lidocaine/

saline injected groups (see below), animals received stimuli until they reached three stage 5 seizures or the maximum number of 22 stimuli had been delivered. In our preliminary experiments, a maximum number of 22 stimulations triggered at least three stage 5 seizures in NEC rats. Therefore, the electrical stimulation was terminated following the 22nd stimulus.

The progression of kindling was evaluated as reported previously[3]: stage 1, behavioral arrest, chewing and eye blinking; stage 2, stage 1+rhythmic head movements and head nodding; stage 3, unilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling and clonic convulsion.

For TH and PRV immunohistochemistry experiments (see below), rats received 6 stimuli by which time all had manifested at least one stage 2 seizure. We have previously shown that 6 kindling stimulations in NECs and GAERS lead to stage 2 seizures (before the development of further kindling stages in NEC and the occurrence of the kindling resistance in GAERS).[26] Thus the experiment was designed to keep all NECs and GAERS at the same seizure stage for them to be comparable at the end of the kindling course for subsequent TH and PRV immunohistochemistry. These rats that received 6 kindling stimulations are referred here as GAERS-STIM and NEC-STIM. Sham-operated NEC (NEC-SHAM) and sham-operated GAERS (GAERS-SHAM) did not have their thresholds determined and never received any kindling stimuli.

Lidocaine injections into the $SNR_{anterior}$ or $SNR_{posterior}$ of GAERS during kindling

To evaluate whether the administration of lidocaine into the $SNR_{anterior}$ or $SNR_{posterior}$ could have any effect on the progress of the kindling, GAERS were pre-treated with bilateral injections of 2% lidocaine in saline (250 nL/per site) or with saline alone (250 nL/per site) over 2 minutes. Thirty minutes after each lidocaine or saline injection, animals were stimulated at the AD threshold until they reached stage 5 or received the maximum number of 22 stimulations.

EEG recording and analysis

The electrical activity of the cortex and of the stimulation site, BLA, was amplified (through a BioAmp ML 136 amplifier) with band pass filter settings at 1- 40 Hz and recorded with a PowerLab 8S System running the Chart v.5 program (ADI Instruments, Oxfordshire, U.K.). An AD lasting two seconds or more immediately after the stimulation was accepted for the threshold determination.[3] The AD duration was taken to be the total duration of ADs on the EEG recorded from the BLA or the cortical electrode following the end of the stimulation period. There were no differences between cortical and amygdaloid AD durations.

The baseline EEG was recorded over 8-h period (between 9.00 am-5.00pm) from all GAERS and NEC animals. For the absence seizure activity of GAERS, a spike-and-wave discharge (SWD) complex was identified if its duration was ≥ 1 s with a train of sharp spikes and slow waves (7.5- 9 Hz) and an amplitude of at least twice the background amplitude of the EEG [4] No absence-like activities were found in NEC animals. The EEG recordings were started 20 min before each lidocaine or saline injections and ended 20 min after each kindling stimulation in GAERS. The sum of the individual durations of all the

SWD complexes present over 10-min periods was used to calculate the cumulative durations of SWDs.

Histological verification

In order to determine the placements of the cannula and BLA electrode, animals were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and decapitated. Brains were then removed and fixed in formalin overnight at 4 °C and then transferred to 30% sucrose in phosphate buffered saline (PBS) (4 °C). When they sank, frozen sections were cut at 40 μm on a cryostat and stained with thionin. Only the data from rats with verified symmetrical bilateral placements in the $SNR_{anterior}$ or $SNR_{posterior}$ and from rats with correct electrode placement were included in the data analysis (Supporting Figure 4, an example of the histological evidence of the cannula positions).

Comparison of TH- and PRV-ir in the $SNR_{posterior}$ or $SNR_{anterior}$ of GAERS and NEC rats: Tissue preparation and immunohistochemistry

Animals were divided into sham and stimulated groups, depending on whether or not they received stimulation. Thus there were four experimental groups each comprised of five rats: NEC-SHAM, GAERS-SHAM, NEC-STIM, and GAERS-STIM.

One hour after the 6th electrical stimulation, rats were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with neutral buffered formalin solution (Sigma). The brains were dissected out and fixed in formalin overnight at 4 °C and then transferred to 30% sucrose in phosphate buffered saline (PBS) (4 °C). When they sank, they were fast frozen in dry ice / 2-isomethylbutane for 3 minutes at -20 °C and stored at -80 °C until further use.

Perfused brains were cut in a cryostat (Microm, GmBH HM520, Germany) to obtain 40 μm sagittal sections (right hemisphere) containing the SNR. Sagittal sections were processed with Nissl staining to confirm the localization and morphology of the SNR and the electrode placements. The characteristic morphology of the SNR in the Nissl-stained sections consisted of an oval structure posterior to the subthalamic nucleus that had a compact layer of cells at the rostrodorsal border, representing the SNC.[16] Adjacent SNR sections were stained with a mouse anti-TH antibody (MAB5280, Millipore, Billerica, MA) and mouse anti-PRV antibody (P3088, Sigma, St Louis, MO). Free-floating sections were incubated in 1% hydrogen peroxide in PBS (30 min, room temperature), and blocking solution (consisted of PBS with 10% normal goat serum (NGS), 0.1% Triton X-100, and 0.1% bovine serum albumin (BSA)) for 1 hour. Subsequently, sections were transferred to a 1.5% NGS, 0.3% Triton X-100, and 0.1% BSA solution in PBS that contained 1:1000 anti-TH or 1:3000 anti-PRV antibody for 1 day (4 °C). Sections were then incubated with an anti-rabbit secondary biotinylated antibody (Vector Laboratories, CA, USA) for 90 min at room temperature, incubated for 1 hour in ABC solution (Vectastain Elite Standard kit, Vector Laboratories, CA, USA) and stained with NovaRED substrate kit. At the end of the staining, sections were washed and mounted with Vectamount on slides.

Densitometry

SNR contains primarily PRV-ir neurons. TH-ir neurons are present in the $SNR_{posterior}$ subregion only.[15] For densitometric measurements, the selection of $SNR_{anterior}$ and $SNR_{posterior}$ subregions were done as described previously.[27,28] Densitometric analysis was done as previously reported.[16,29] Briefly, photomicrographs (252×) of optical fields corresponding to the $SNR_{anterior}$ and $SNR_{posterior}$ were captured via an Olympus DP72 microscope (Tokyo, Japan), and transferred via an Olympus U-TV0.63XC Digital Imaging Camera (Tokyo, Japan) to a computer. Signal densitometry of PRV- and TH-ir SNR neurons was done with the Image J software (Wayne Rasband, NIMH, MD, USA) to obtain a semiquantitative measure of difference in the expression of TH-ir and PRV-ir. To measure PRV-ir, two optical fields per SNR section were selected to represent $SNR_{anterior}$ and $SNR_{posterior}$. Measurement of TH-ir was done in $SNR_{posterior}$. All densitometric measurements were performed in the same SNR subregions using the same light intensity and camera settings using Image J (Wayne Rasband, NIH, USA). The cell bodies of 10-20 neurons were sampled to estimate the mean cellular TH-ir or PRV-ir for each section (see Supporting Information). Mean background densitometric values were obtained from regions in the same section that had no apparent staining. An average of 4–5 SNR sections per rat was included. The background-subtracted mean densitometric TH-ir or PRV-ir values of these sections were averaged for each brain and results were used in the statistical analysis. To avoid interassay variability, all values were referred as percentage of PRV-ir or TH-ir in the NEC-SHAM group, which was assayed in parallel with the other experimental groups.

The number of TH-ir or PRV-ir neurons were also counted. We did not find any differences in terms of TH-ir or PRV-ir cell number among NEC-SHAM, GAERS-SHAM, NEC-STIM and GAERS-STIM groups (see Supporting Information).

Statistics

Statistics were done with Prism (GraphPad Software, CA, USA). Data were statistically evaluated by analysis of variance of repeated measures (ANOVA). A two-way ANOVA followed by the post hoc Bonferroni test was used to analyze the kindling rate and AD duration of GAERS pretreated with lidocaine or saline into the $SNR_{anterior}$ or $SNR_{posterior}$ regions (*2 factors "Group" and "Number of stim")*. A two-way ANOVA followed by the post hoc Bonferroni test (2 *factors "Strain" (NEC vs GAERS) and "STIM" (kindling vs sham)* was used to analyze the intensity of PRV-ir or TH-ir in the $SNR_{posterior}$ or $SNR_{anterior}$ among NEC-SHAM, GAERS-SHAM, NEC-STIM and GAERS-STIM. A one-way ANOVA followed by the post hoc Dunnett test was used to compare the mean AD thresholds and the mean number of stimulations for the development of the first stage 2 among the GAERS pretreated with lidocaine or saline into the $SNR_{posterior}$ or $SNR_{anterior}$. The level of statistical significance was considered to be $p<0.05$.

Results

Lidocaine injections into the $SNR_{posterior}$ eliminate the resistance to kindling in GAERS

All of the GAERS pretreated with lidocaine injections into the $SNR_{posterior}$ reached stage 5 following kindling stimulations, whereas of the animals pretreated with lidocaine into the $SNR_{anterior}$ all failed to reach stages 3, 4, or 5 and stayed at stage 2 (Fig. 1A, Table 1). Two-way ANOVA with repeated measures analysis revealed significant differences among groups (Group effect: F=67.95, DFn=3, DFd=567, $p<0.001$). GAERS pretreated with saline in both $SNR_{anterior}$ and $SNR_{posterior}$ groups stayed at stage 2 seizures even after 22 stimulations. The mean AD thresholds did not show any differences among the GAERS pretreated with lidocaine or saline into the $SNR_{posterior}$ or $SNR_{anterior}$ (Table 1). The mean of the AD durations following the stimulations showed significant differences among groups (Group effect: F= 11.99, DFn= 3, DFd= 567, $p<0.001$) (Fig. 1B). Lidocaine injections into the $SNR_{anterior}$ decreased the AD durations in 9th, 12nd, 19th and 20th stimulations compared to those in lidocaine injected $SNR_{posterior}$ group. Post-hoc Bonferroni test also revealed significant differences between lidocaine injected $SNR_{anterior}$ vs saline injected $SNR_{anterior}$ groups in 12nd, 18th, 19th, 21st and 22nd stimulations.

Lidocaine or saline injections into the $SNR_{anterior}$ or $SNR_{posterior}$ did not affect cumulative durations of SWDs in GAERS

In order to evaluate whether lidocaine or saline injections into the $SNR_{anterior}$ or $SNR_{posterior}$ affects the occurrence of spike-and-wave discharges (SWDs) in GAERS, we measured the cumulative durations of SWDs before and after each injections. Fig. 2 shows the average of the cumulative durations of SWDs using from the 2^{nd} to the 20^{th} stimulations in either lidocaine or saline injections into the $SNR_{anterior}$ or $SNR_{posterior}$ of GAERS. Repeated measures of two-way ANOVA followed by post hoc Bonferroni test did not reveal significant differences among pre- and post-injection and post-stimulation periods or among groups.

Cellular TH-ir in the $SNR_{posterior}$ in both NEC and GAERS and cellular PRV-ir in the $SNR_{posterior}$ in NEC increased by kindling stimulations

For the comparison of the densitometry results, a repeated-measures ANOVA design with 2 factors “Strain” (2 levels: NEC vs GAERS) and “STIM” (2 levels: kindling vs sham) was applied to both TH-ir and PRV-ir results, separately. TH-ir was measured semiquantitatively with immunohistochemistry in the $SNR_{posterior}$ region only, since no dopaminergic neurons were present in the $SNR_{anterior}$. There were no significant differences in mean cellular TH-ir between NEC and GAERS groups (Strain effect: F= 0.06, DFn= 1, DFd= 74, p=0.804) (Fig. 3). However, cellular TH-ir expression in $SNR_{posterior}$ significantly increased by 6 stimulations of BLA in both NEC and GAERS group compared to the NEC-SHAM and GAERS-SHAM rats (STIM effect: F= 16.92, DFn= 1, DFd= 74, $p<0.0001$). There was no significant interaction between Strain and STIM factors (Interaction: F= 0.02, DFn=1, DFd=74, p=0.878).

We compared densitometrically the PRV-ir in the $SNR_{posterior}$ or $SNR_{anterior}$. We used PRV rather than GAD as a marker of SNR GABAergic interneurons, because it demonstrates a

stronger perisomatic expression than GAD, offering a more reliable measure of cellular expression. There were no significant differences in mean cellular PRV-ir in $SNR_{posterior}$ or $SNR_{anterior}$ between NEC-SHAM and GAERS-SHAM groups (Strain effect for $SNR_{anterior}$ F=1.06, DFn=1, DFd=44, p=0.309. Strain effect for $SNR_{posterior}$ F=0.85, DFn=1, DFd=42, p=0.361). In the $SNR_{posterior}$, PRV-ir expression significantly increased in NEC-STIM group compared with NEC-SHAM (STIM effect: F= 10.66, DFn= 1, DFd= 42, p=0.002). However, kindling stimulation did not alter PRV-ir in the $SNR_{anterior}$ of GAERS-STIM group (STIM effect: F= 0.36, DFn= 1, DFd= 44, p=0.55) (Fig. 4). While there was no significant interaction between Strain and STIM factors in $SNR_{anterior}$ (Interaction F: 0.05, DFn=1, DFd=44, p=0.821), this interaction was significant (Interaction F: 4.51, DFn=1, DFd=42, p=0.0396) in $SNR_{posterior}$.

The mean PRV-ir and TH-ir cell numbers of SNR in the NEC-SHAM, GAERS-SHAM, NEC-STIM and GAERS-STIM groups were given in Supporting Information, Supporting Figure 2 and 3.

Discussion

The main findings of the present study are as follows: (1) All GAERS pretreated with lidocaine injections into the $SNR_{posterior}$ progressed to stage 5 in the kindling course, whereas GAERS animals pretreated with lidocaine into the $SNR_{anterior}$ did not go beyond stage 2 with the maximum number of stimulations and thus failed to the progress of kindling, (2) NEC-SHAM and GAERS-SHAM rats showed no significant differences in TH-ir and PRV-ir expressions in the $SNR_{anterior}$ or $SNR_{posterior}$, (3) Cellular TH-ir expression in the $SNR_{posterior}$ significantly increased by 6 kindling stimulations in both NEC and GAERS groups compared to the sham operated rats, (4) Six kindling stimulations (stage 2) increased the cellular PRV-ir expression only in the the $SNR_{posterior}$ of NEC group.

One of the most interesting findings of this study is that $SNR_{posterior}$ is a key regulator of kindling resistance in GAERS, via a lidocaine sensitive process. The focal application of lidocaine in the $SNR_{posterior}$ eliminated the resistance to stage 3-5 seizures and all GAERS in the $SNR_{posterior}$ group experienced stage 5 generalized motor seizures. However, lidocaine infusions in the $SNR_{anterior}$ failed to alter the kindling resistance in GAERS. The SNR, a main basal ganglia output structure, is thought to be crucially involved in the control of seizures, affecting either the latency to seizure onset or their propagation, in a region, age, and sex specific manner[23]. In kindling induced seizures, the SNR controls the transition from limbic to generalized motor seizures.[30-32] In the SNR of adult male rats, infusions of $GABA_A$ receptor agonists in the $SNR_{anterior}$ and $SNR_{posterior}$ subregions mediate separate inhibitory or facilitatory effects respectively on the onset, propagation and/or termination of generalized seizures.[11,33] Veliskova et al.[34] found that in the flurothyl-induced generalized seizures in rats, the $SNR_{posterior}$ subregion was selectively active during the pre-clonic period while the $SNR_{anterior}$ was not involved at this stage. They proposed the $SNR_{posterior}$ as an early gateway to seizure propagation. In the present study, the elimination of the kindling resistance by lidocaine infusions in the $SNR_{posterior}$ of GAERS is in line with reports showing the region-specific regulation of seizures by the SNR and the specific role of the $SNR_{posterior}$ subregion in the secondary generalization of focal limbic seizures. The

effects of lidocaine infusions in this study are similar to those described for muscimol, suggesting that silencing of the activity of $SNR_{posterior}$ neurons may mediate the observed proconvulsant effects. Moreover, the lidocaine injections into the $SNR_{anterior}$ decreased the AD duration, suggesting a possible anticonvulsant effect with the $SNR_{anterior}$ infusions. This result is supported by previous studies, showing anticonvulsant effect of the bilateral microinfusions of muscimol into the $SNR_{anterior}$.[23]

Six kindling stimulations selectively increased PRV-ir in the $SNR_{posterior}$ of the NEC-STIM group but not in GAERS-STIM. PRV is a calcium buffering protein that controls intracellular activity-dependent and calcium-sensitive processes and neuronal firing.[35] The increase in PRV-ir may reflect the propagation of the seizure discharge in the $SNR_{posterior}$ which occurs during the transition from focal to generalized seizures during kindling in NEC rats.[36] The failure of the kindling resistant GAERS-STIM rats to manifest the stimulation-induced increase in PRV-ir could indicate that the seizure discharges may not stimulate the GABAergic $SNR_{posterior}$ neurons sufficienty to increase PRV-ir in the GABAergic $SNR_{posterior}$ neurons. Kindling stimulus induced increase of PRV-ir in the GABAergic $SNR_{posterior}$ neurons may therefore be an early marker of propensity to express generalized seizures, providing a testable hypothesis of the mechanism underlying the kindling-resistance of GAERS.

There are no prior studies known to us on the effects of kindling stimulation on PRV-ir in the SNR. Interestingly, increased numbers of PRV-ir interneurons in the dentate gyrus of kindled mice were observed in mice that exhibited greater post-kindling reduction in AD threshold.[37] In other stimulation paradigms however, like theta burst stimulation, decrease in PRV-ir has been shown after stimulation, although this effect seemed to depend on the timing and pattern of stimulation.[38]

The STIM-induced increase in TH is unlikely to underlie the kindling resistance on its own, since it is present in both NEC and GAERS stimulated rats. This possibility is in line with the findings of Albala et al[39] who showed that the selective destruction of the nigrostriatal dopaminergic neurons does not modify amygdaloid kindling in adult rats. In agreement, other studies showed that the number of stimulations to reach kindling convulsions, the latency to onset of convulsions or AD durations were unaffected by the dopamine depletion.[40] However, the current data cannot exclude that stimulation-induced changes in dopaminergic $SNR_{posterior}$ neurons could contribute to the effects of GABAergic neurons in kindling or kindling resistance. Other studies have reported increase or decrease in TH-ir in the hippocampus or amygdala of kindled animals.[41,42] These varied findings may indicate that the brain region and cell type, or the timing after stimulation may be important in the effects of kindling on TH-ir.

A lack of changes in GAERS can be considered as a “cell-type-specific-response” and suggest that GAERS rats showing kindling resistance, manifest cell type specific responses to 6 kindling stimulations during the period that precedes the time when kindling resistance can be documented. The cell type specific $SNR_{posterior}$ responses to 6 kindling stimuli in GAERS in the absence of overt kindling differences suggest that cell type specific responses precede the expression of kindling resistance.

In basal conditions, no significant difference in TH-ir and PRV-ir in the $SNR_{anterior}$ and $SNR_{posterior}$ subregions was demonstrated in the control NEC rats and GAERS indicating that the strain differences in kindling resistance are due to stimulation-specific responses rather than pre-existing differences in these cellular markers.

Pharmacological potentiation of $GABA_A$-mediated transmission, within the SNR, by bilateral microinjections of muscimol, a $GABA_A$ agonist, suppresses the SWDs in GAERS[20] but lidocaine infusions in the $SNR_{anterior}$ or $SNR_{posterior}$ do not. These indicate different functional effects on SNR mediated control of SWDs in GAERS by $GABA_A$ receptor agonists or sodium channel inhibitors. Further studies are needed to investigate whether these are dose-dependent differences that are due to altered cell-type specific expression or subcellular distribution of these channels in GAERS. In addition, muscimol was administered into the whole SNR in the above-mentioned studies, whereas in the present study, lidocaine was injected into the $SNR_{anterior}$ or $SNR_{posterior}$ subregions. Thus, this is the first study reporting insensitivity to lidocaine on the occurrence of SWDs in the $SNR_{anterior}$ or $SNR_{posterior}$ of GAERS.

To our knowledge, the present study is the first report of TH-ir and PRV-ir in the subregions of the SNR in GAERS, presenting region-, cell-type and strain-specific differences in their expression during kindling. The present study supports the prior literature that the SNR is not a uniform structure concerning its role as a seizure modulation gate. Specifically, the $SNR_{posterior}$ is a site that is crucially involved in the resistance to the transition from limbic to generalized motor seizures in GAERS in the kindling model via a lidocaine sensitive manner. The stimulation-induced expression changes in TH-ir and PRV-ir in the $SNR_{posterior}$ confirm the importance of cell-type specific responses during the kindling stimulations and implicate the GABAergic $SNR_{posterior}$ neurons in kindling resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK), Project No: 111S209 and Marmara University Research Council. SLM received research grants from NINDS (NS078333, NS020253), U.S. Department of Defense (W81XWH-13-1-0180), CURE, UCB, the Heffer Family and Barry Segal Family Foundations and the Abbe Goldstein/Joshua Lurie and Laurie Marsh/Dan Levitz families. ASG received research grants from NINDS (NS078333, NS091170), U.S. Department of Defense (W81XWH-13-1-0180), CURE, UCB, the Heffer Family and Barry Segal Family Foundations and the Abbe Goldstein/Joshua Lurie and Laurie Marsh/ Dan Levitz families.

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Key Bullet Points

1. GAERS pretreated with lidocaine injections into the $SNR_{posterior}$ progressed to stage 5 in the kindling course
2. GAERS animals pretreated with lidocaine into the $SNR_{anterior}$ did not go beyond stage 2 with the maximum number of stimulations and thus failed to the progress of kindling
3. The $SNR_{posterior}$ is involved in the resistance to generalized motor seizures in genetic absence epilepsy rats.
4. There is no strain effect in TH-ir and PRV-ir expression in the $SNR_{anterior}$ and $SNR_{posterior}$ of naïve non-epileptic and epileptic rats.

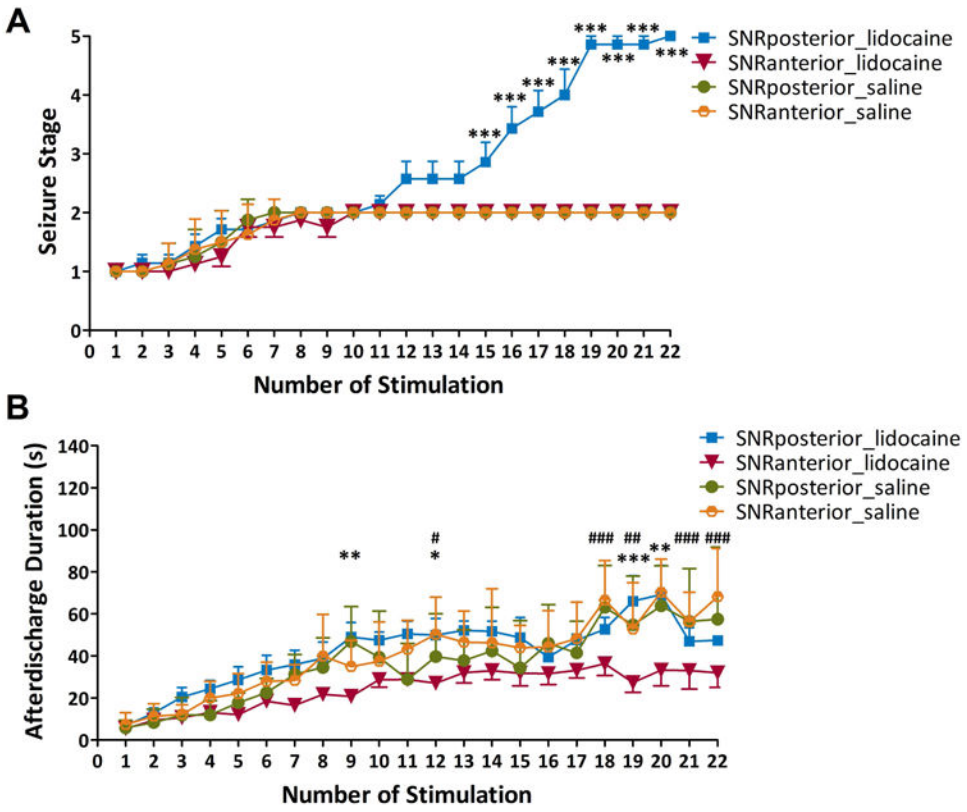

Figure 1.

Seizure stages and AD durations in the ipsilateral BLA of GAERS that was injected either with lidocaine or saline 30 min before each kindling stimulation. (A) GAERS pretreated with lidocaine injections into the $SNR_{posterior}$ (n=7) reached stage 5 after repeated kindling stimulations, whereas all the animals pretreated with lidocaine into the $SNR_{anterior}$ (n=8) failed to reach stages 3, 4, or 5. Saline injections either into $SNR_{posterior}$ (n=8) or $SN_{anterior}$ (n=8) did not affect the kindling resistance in GAERS. (B) Lidocaine injections into the $SNR_{anterior}$ decreased the AD durations. Asterisks indicate significant differences between lidocaine injected ($SNR_{posterior}$ *vs* $SNR_{anterior}$) groups. Pound (#) signs indicate significant differences between lidocaine injected $SNR_{anterior}$ and saline injected $SNR_{anterior}$ groups. Data are expressed as mean±S.E.M ***p<0.001, **p<0.01, *p <0.05, $^{###}$p<0.001, $^{##}$p<0.01, $^{#}$p<0.05.

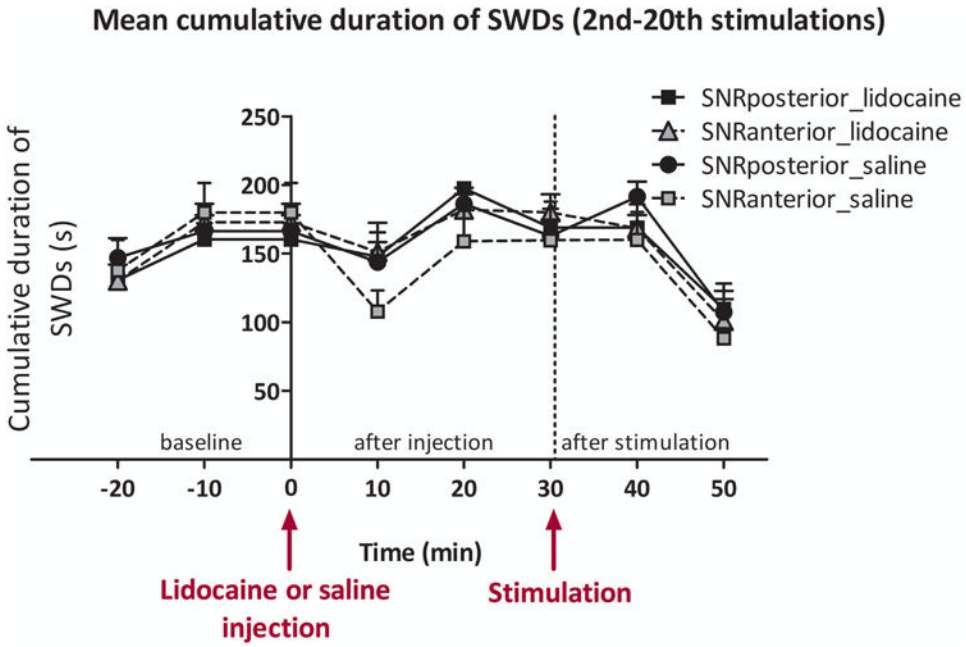

Figure 2.

The average of the mean cumulative durations of SWDs (from 2nd to 20th stimulations recordings) was given in the pre- and post-injection and post-stimulation periods of either lidocaine or saline injections into the $SNR_{anterior}$ or $SNR_{posterior}$ of GAERS. Repeated measures of two-way ANOVA followed by post hoc Bonferroni test did not reveal significant differences among pre- and post-injection and post-stimulation periods among groups. Data are expressed as mean±S.E.M.

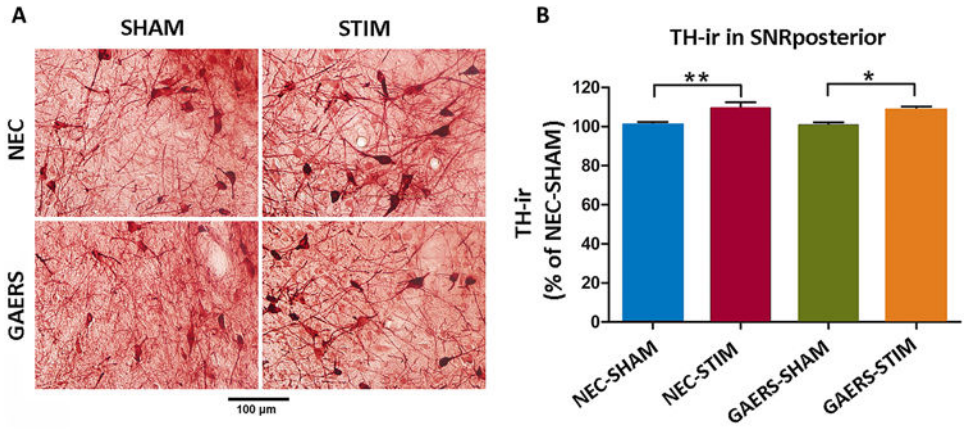

Figure 3.

The effect of kindling stimulations on TH-ir in $SNR_{posterior}$ of NEC and GAERS. A) Representative photomicrographs of $SNR_{posterior}$ of either sham- or stimulated- (STIM) NECs and GAERS stained with TH-specific immunochemistry. B) Six electrical stimulations of BLA increased TH-ir both in NEC and GAERS (n=5, each group). Results are expressed as percentage of cellular TH-ir in NEC-SHAM $SNR_{posterior}$ neurons, *$p<0.05$.

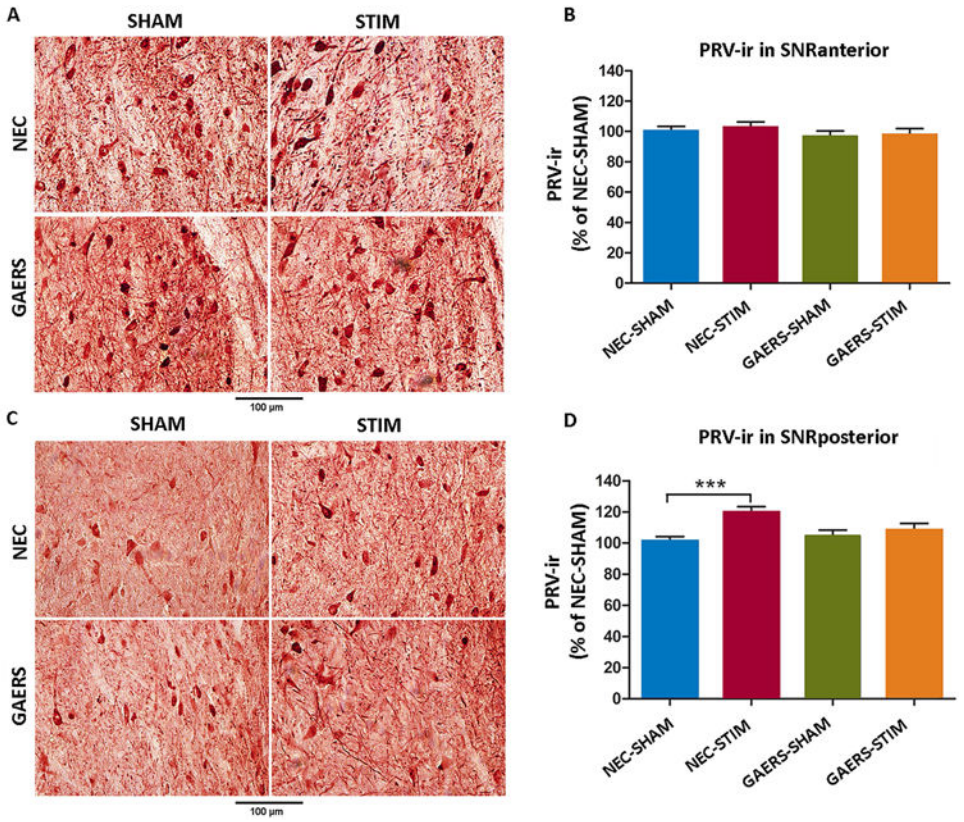

Figure 4.

The effect of kindling stimulations on PRV-ir in $SNR_{anterior}$ and $SNR_{posterior}$ of NEC and GAERS. A) Representative photomicrographs of $SNR_{anterior}$ of either sham- or STIM NECs or GAERS stained with PRV-specific immunochemistry. B) Six electrical stimulations of BLA did not affect PRV-ir in NEC or GAERS groups compared with sham operated rats (n=5, each group). C) Representative photomicrographs of $SNR_{posterior}$ sections of either sham or stimulated NECs or GAERS stained with PRV-specific immunochemistry. D) Six electrical stimulations of BLA increased PRV-ir in NEC group compared with sham operated rats but had no effect on PRV-ir of GAERS. Results are expressed as percentage of cellular PRV-ir in NEC-SHAM $SNR_{anterior}$ or $SNR_{posterior}$ neurons (n=5, each group). ***$p<0.001$.

Table 1

The mean AD thresholds and the mean number of stimulations for the development of the first stage 2-5 seizures in the GAERS pretreated with lidocaine or saline into the $SNR_{posterior}$ or $SNR_{anterior.}$ A one-way ANOVA followed by post hoc Dunnet test did not reveal significant differences in the mean AD thresholds or in the mean number of stimulations for the development of the first stage 2 among the 4 groups. Data are expressed as means±S.E.M.

			The mean number of stimulations for the development of the first stage 2—5 seizures			
Groups	**Number of rats (n)**	**AD threshold (μA)**	**stage 2**	**stage 3**	**stage 4**	**stage 5**
$SNR_{posterior}$_lidocaine	7	114.3±17.98	5.00±0.75	13.67±0.91	16.29±0.80	18.7±10.6
$SNR_{anterior}$_lidocaine	8	118.8±12.27	6.75±0.86	-	-	-
$SNR_{posterior}$_saline	8	91.25±6.39	5.25±0.45	-	-	-
$SNR_{anterior}$_saline	8	96.25±12.68	5.50±0.62	-	-	-