

# The effect of amygdala kindling on neuronal firing patterns in the lateral thalamus in the GAERS model of absence epilepsy

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## SUMMARY

**Objective:** The co-occurrence of absence and mesial temporal lobe epilepsy is rare in both humans and animal models. Consistent with this, rat models of absence epilepsy, including genetic absence epilepsy rats from Strasbourg (GAERS), are resistant to experimental temporal lobe epileptogenesis, in particular by amygdala kindling. Structures within the cortical-thalamocortical system are critically involved in the generation and maintenance of the electrographic spike-and-wave discharges (SWDs) that characterize absence seizures. Using *in vivo* electrophysiologic recordings, this study investigated the role of thalamocortical circuitry in the generalization of amygdala-kindling induced seizures in the GAERS and the nonepileptic control (NEC) strain of Wistar rats.

**Methods:** GAERS and NEC rats were implanted with a stimulating electrode in amygdala and stimulated at afterdischarge threshold twice daily to a maximum number of 30 stimulations. Thereafter extracellular single neuron recordings were performed *in vivo* under neuroleptanesthesia in the thalamocortical network.

**Results:** In NEC rats, amygdala kindling induced convulsive class V seizures and altered characteristics of neuronal activity in the thalamic reticular nucleus (TRN), in particular decreased firing rates and increased burst firing patterns. Less marked changes were seen in other regions examined: the ventroposteromedial nucleus of thalamus (VPM), the CA3 region of the hippocampus, and the deep layers (V/VI) of the cortex. GAERS did not progress beyond class II seizures, with a matched number of kindling stimulations, and the thalamic neuronal firing alterations observed in NEC rats were not seen.

**Significance:** These data suggest that the TRN plays an important role in kindling resistance in GAERS and is central to the control of secondary generalization of limbic seizures.

**KEY WORDS:** Amygdala kindling, Absence epilepsy, Ventrobasal thalamic nucleus, *In vivo* juxtacellular single unit recordings, Mesial temporal lobe epilepsy.



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Genetic absence epilepsy rats from Strasbourg (GAERS) are a well-validated model of human genetic generalized epilepsy with absence seizures, possessing electrophysiologic, behavioral, and pharmacologic profiles similar to those in the human condition.<sup>1,2</sup> Consistent with clinical observations, it is uncommon for absence seizures and mesial temporal lobe epilepsy to be present in the same patient.<sup>3,4</sup> GAERS display resistance to the progressive development of convulsive limbic seizures induced by amygdala kindling, and the frequency and duration of the absence-associated spike-and-wave discharges (SWDs) remain unchanged.<sup>5–7</sup> This resistance, specifically an inability to progress beyond class II (nonconvulsive) seizures in GAERS, is also a property of other rat models of genetic absence epilepsy.<sup>8</sup> The resistance to generalization of limbic seizures increases with age, and is not present at early ages before SWDs are detected on cortical electroencephalography (EEG).<sup>9</sup> The presence of absence seizure activity is therefore thought to prevent, or significantly retard, the secondary generalization of the limbic seizures from engaging widespread cortical and subcortical networks. This resistance is also seen for other methods of inducing limbic epileptogenesis, such as hippocampal kindling, perirhinal kindling, and post-kainic acid status epilepticus, although the cellular mechanisms underlying this remain undetermined.<sup>8,10,11</sup>

The cortical-thalamocortical (CTC) circuitry is known to play a critical role in the initiation and propagation of SWDs in human absence seizures, and in animal models.<sup>12,13</sup> Increased synchronization and  $\gamma$ -aminobutyric acid (GABA)ergic inhibition in CTC circuits—in particular the ventral basal thalamus (VB) reticular thalamic nucleus (TRN), and the primary and secondary somatosensory cortex—are implicated in the generation of SWDs.<sup>14–17</sup> Neuronal connections between the ventrolateral thalamus and somatosensory cortex are reciprocal with additional collateral projections to TRN.<sup>18</sup> Outputs from the TRN appear to be critical in the resistance to the generalization of limbic seizures.<sup>19</sup>

Low-voltage-activated T-type calcium channels within this circuit mediate burst firing and oscillatory behavior and dysfunction of these channels, with the  $Ca_v3.2$  (Cacna1h) subtype particularly implicated in absence epilepsy pathogenesis.<sup>20–22</sup> The  $Ca_v3.2$  *R1584P* genetic mutation is the only genetic variant shown to date to be associated with the absence epilepsy phenotype in GAERS.<sup>22</sup> The inhibition of low threshold T-type calcium currents in thalamocortical network by the systemic or intracortical administration of the T-type calcium channel blocker, ethosuximide, reduced the kindling resistance, with suppression of SWDs in adult GAERS.<sup>23</sup> Furthermore, in young GAERS, because the CTC circuitry is immature, bilateral intracortical administration of ethosuximide completely abolished the kindling resistance.<sup>24</sup>

These findings raise the possibility that the functionally intact CTC circuit is critical in the secondary generalization

of limbic seizures, and a pathologic CTC circuitry in absence epilepsy contributes to the resistance limbic seizure generalization. Using *in vivo* electrophysiologic recordings, this study aimed to investigate the role of the thalamocortical circuit in limbic seizure generalization by comparing the neuronal firing properties within the CTC circuitry in GAERS and their nonepileptic control (NEC) Wistar rats, before and after amygdala-kindling stimulations. NEC rats were derived from the same original colony as the GAERS, but have been selectively inbred so as to not express SWDs.<sup>1,2</sup>

## METHODS

### Animals

Adult male NEC Wistar rats ( $n = 30$ ) and GAERS ( $n = 30$ ) were used. Rats were 3–6 months old and weighed 250–350 g at the start of the experiments. Animals were maintained under standard laboratory conditions on a 12/12-h light/dark cycle, with food and water available *ad libitum*. The study was approved by the animal ethics committee of the Royal Melbourne Hospital, the University of Melbourne (Ethics number: 0706287), and conformed to Australian National Health and Medical Research Council Guidelines for the ethical use of animals in scientific research. All efforts were made to minimize stress and the number of animals necessary to produce reliable data.

### Stimulating electrode implantation

Rats were anesthetized with ketamine (100 mg/kg, *i.p.*, Ketazol; Richter Pharma, Wels, Austria) plus xylazine (10 mg/kg, *i.p.*, Rompun; Bayer Leverkusen, Germany) and placed into a stereotaxic frame (Stoelting Co. Model 51600; Wood-Dale, IL, U.S.A.). A single midline incision was made on the scalp, and a bipolar stimulating electrode (-MS303/1 twisted; Plastics One Inc., Roanoke, VA, U.S.A.) was stereotaxically implanted in the left basolateral nucleus of the amygdala (BLA) (coordinates: anteroposterior [AP]  $-2.6$  mm, lateral [L] 4.8 mm from bregma, dorsoventral [DV]  $-8.5$  mm from the surface of the skull).<sup>25</sup> For unilateral cortical EEG recording, screw electrodes were placed on the dura over the left frontal (AP  $+2.0$  mm, L 3.5 mm from bregma) and left occipital cortex (AP  $-6.0$  mm, L 4.0 mm from bregma).<sup>25</sup> A ground electrode was placed over the cerebellum. Recording electrodes comprised a 1.3-mm male connector (Farnell Components, Chester Hill, NSW, Australia) soldered onto a nickel alloy jeweler screw. All electrodes were fixed in position by applying dental cement (Vertex; Vertex-Dental, Zeist, The Netherlands) around and only over the left hemisphere of the skull to leave the bregma and right hemisphere intact for electrophysiologic recordings. The incision was then sutured (Dysilk/3.0). Rats were allowed a 1-week recovery period before the start of the EEG recording and kindling procedure.

### Electrical amygdala kindling

After the recovery period, electrical stimulations were delivered from an isolated constant current stimulator (Accupulser A310, Stimulus isolator A365; World Precision Instrument, Sarasota, FL, U.S.A.) applied through the bipolar electrode (monophasic square-wave current; frequency = 80 Hz; duration = 2 s; pulse width = 1 msec) to the BLA. For the rats in the stimulated group, afterdischarge threshold (ADT) was defined as the minimal intensity able to trigger an autonomous spike discharge in the EEG lasting  $\geq 6$  s immediately after the amygdala stimulation.<sup>26</sup> The first ADT (100–400  $\mu$ A) was determined by increasing the current intensity by 50  $\mu$ A steps until an afterdischarge was observed. Then the rats in the stimulated groups received electrical stimulations at their ADT current intensity twice daily, with an interstimulation interval of at least 4 h, 5 days per week until a total of 30 stimulations (i.e., 3 weeks) (Fig. S2C). Behavioral manifestations of the seizures induced by the electrical stimulations were classified according to the stages described by Racine<sup>27</sup>: class I, facial clonus; class II, head nodding; class III, contralateral forelimb clonus; class IV, bilateral forelimb clonus and rearing; and class V, rearing and falling. Afterdischarge lengths were assessed from the EEG recordings off line. Following completion of kindling, *in vivo* electrophysiologic recordings were performed—these commenced on the day of, and at least 1 h after, the last (30th) kindling stimulation. Rats in the unstimulated groups underwent the same surgeries and handlings but did not receive any electrical stimulation.

### In vivo extracellular recordings

Initially a series of surgical procedures were performed for preparation of *in vivo* extracellular recordings (see Data S1). Starting at least 1 h after completion of all surgical procedures, the interictal patterns of single neurons in layers IV/V of the primary somatosensory cortex, the CA3 pyramidal layer of hippocampus, and thalamic nuclei (TRN and ventroposteromedial nucleus of the thalamus [VPM]) were examined by *in vivo* extracellular single-unit recordings under fentanyl-haloperidol neuroleptanesthesia.<sup>28</sup> All electrophysiologic recordings were performed simultaneously with surface EEG to monitor the stability of anesthesia. In addition, we recorded simultaneous local field potentials from the amygdala through the implanted depth electrode and from the TRN through the glass micropipettes.

Micropipettes were prepared from 1.5-mm glass capillaries (30-0020; Harvard Apparatus Ltd., Kent, United Kingdom) on a vertical puller (Sutter Instruments, Novato, CA, U.S.A.). They were filled with a 1.5% solution containing *N*-(2-aminoethyl) biotinamide hydrochloride (Neurobiotin; Vector Laboratories, Burlingame, CA, U.S.A.) dissolved in 1 M potassium acetate (CH3COOK). The pipette tips were broken to a diameter of  $\sim 1$   $\mu$ m. Two micropipettes (resistance, 30–50 M $\Omega$ ) were clamped onto holders attached to motorized stereotaxic microdrivers (StereoDrive, Neuro-

star, Germany) and slowly lowered through a small burr hole (diameter  $< 0.8$  mm), targeting TRN and VPM, respectively, of the right hemisphere contralateral to the kindling electrode in the BLA. Stereotaxic coordinates (measured from bregma) were as follows: TRN (AP  $-2.8$  mm, L 4.8 mm, DV 6.0 mm from dura, 12.5 degree angle) and VPM (AP  $-3.6$  mm, L 3.4 mm, DV 5.0 mm from dura, 0 degree angle) (Fig. S1A). Pyramidal cells were recorded within the layers IV or V of the primary somatosensory cortex (AP  $-3.6$  mm, L 3.4 mm, DV 0.5–1.5 mm under the cortical surface) and within the CA3 pyramidal layer of hippocampus (AP  $-3.6$  mm, L 3.4 mm, DV 2.5–3.2 mm under the cortical surface) as shown in Fig. S1A.<sup>25</sup>

### Analysis of in vivo extracellular recordings

The extracellular neuronal activity was recorded and recordings were analyzed with the pClamp 10 (Axon Instruments, Foster City, CA, U.S.A.). Signals were digitized at a sampling rate of 20 kHz per channel (Digidata 1200B; Axon Instruments) using pCLAMP 10 (Axon Instruments). Data were processed (Cyber-Amp 380; Axon Instruments) with band passes of 0.1–1,200 Hz for the EEG and 0–6,000 Hz for cellular activity. All recorded neurons exhibit action potentials (APs) that can occur as either single APs or as bursts of AP. A burst is considered to be two or more APs firing within 6 msec of each other, measured from the onset of each AP. Neuronal firing patterns were recorded for at least 15 min. Then for the analysis of neuronal firing patterns, these recordings were divided into three segments; from each segment, 60 s samples were selected. Because GAERS experience absence seizures, the selection of 60 s samples was screened to ensure they contained no SWD activity, and the firing patterns during these samples therefore represent interictal activity. Ictal periods were then identified as a SWD activity with the duration of  $\geq 1$  s with a train of sharp spikes and slow waves (7.5–9 Hz) and amplitude of at least twice the background amplitude of the surface EEG of rats in neuroleptanalgesia. In NEC animals, no SWD activity was recorded and the neuronal firing patterns were analyzed accordingly. The mean neuronal firing frequency, percentage of burst, the mean number of APs per burst, the maximum number of APs per burst and intraburst firing frequency for each segment were evaluated interictally and averaged for each cell. The identification of pyramidal and nonpyramidal neurons is based on the characteristics of individual action potential complexes.<sup>29</sup> The spike width of  $< 0.5$  msec at half amplitude was considered as a nonpyramidal, presumably GABAergic interneuron, which is shorter than the spike width in pyramidal neurons.<sup>30</sup>

In addition, we performed cross-correlation analysis on the simultaneous local field potentials between the TRN and amygdala to examine the degree of synchrony between these regions before and after kindling in GAERS and NEC rats. For all of the cross-correlations, the 30 s period before

and 30 s after the stimulation were chosen. The cross-correlation is plus/minus 2 s with bin widths of 20 msec, and it is always TRN versus amygdala.

At the end of the recording sessions, neurobiotin was electrophysiologically applied to the last pair of recorded neurons using single-cell labeling methods to identify the location of the recorded cell according to the method described by Pinault<sup>31</sup> (see Data S1, Fig. S1).

### Euthanasia and transcardial perfusions

After the labeling procedure, the animal was given an overdose of pentobarbital (50 mg/kg i.v.) (Lethobarb; Virbac, Milperra, NSW, Australia) and transcardially perfused with phosphate-buffered saline (PBS, 10 mM, 0.9% NaCl, pH 7.45; 200 ml) followed by 500 ml of a fixative containing 4% paraformaldehyde (PFA) in 10 mM PBS. The brain was then extracted and post fixed in 4% PFA for 24 h.

### Histology

The fixed brains were immersed in PBS and sectioned on a vibratome (Vibratome, St Louis, MO, U.S.A.). Coronal brain sections were cut at 100  $\mu$ m and serially collected in PBS. To reveal the tracer injected into the recorded neurons, we used nickel-intensified 3,3P-diaminobenzidine tetra hydrochloride (DAB), using the kit substrate peroxidase DAB (Vector Laboratories) (see Data S1).

### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc.; La Jolla CA, U.S.A.). The Mann-Whitney *U* test was used for comparison of nonnormally distributed continuous variables of interictal neuronal firing patterns between groups/within groups, and for this reason median value was used for expression. Two-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test was used to compare the kindling rate and afterdischarge duration between the groups. *p*-Values of <0.05 were considered statistically significant. Correlations between the firing frequency and burst firing were tested using Pearson's correlation analysis.

## RESULTS

### GAERS are resistant to the progression of amygdala kindling

Consistent with previous studies, all NEC rats reached class V seizures during kindling; however, 14 of 15 GAERS did not progress beyond class II seizures after 30 stimulations (Fig. S2A). One GAERS exhibited class IV seizures by the 27th stimulation, but did not progress to class V (see Fig. S2A)—this animal was excluded from single cell recording experiments. Afterdischarge durations were longer in NEC rats compared to GAERS (Fig. S2B; *F* value: 1.279, *p* < 0.01), reaching post hoc significance at the 12th stimulation, at which time NEC rats were experiencing class III seizures. The mean ADT prior to kindling was  $182.5 \pm 6.7 \mu$ A in the NEC rats and  $193 \pm 12.3 \mu$ A in the GAERS (*p* = 0.39). A sample EEG trace from cortex and amygdala showing the afterdischarges after 2 s amygdala kindling stimulation is presented in Figure S2C. The mean frequency of kindling-induced seizures in NEC and GAERS was around 4 Hz and did not change significantly with repeated stimulations. At the 30th stimulation, GAERS showed a slight increase in frequency ( $6.6 \pm 0.9$  Hz); however, this increase was not significant when compared to NEC (Fig. S2D). The mean duration of limbic and convulsive seizures in NEC and GAERS is presented in Figure S2E. In NEC rats the limbic seizure duration decreased and the convulsive seizure duration increased with repeated stimulations; however, in GAERS, limbic seizure duration showed a tendency to increase after the 20th stimulation and there were no convulsive seizures (Fig. S2E).

### The interictal firing patterns in VPM and somatosensory cortex differ between the unstimulated-NEC rats and unstimulated GAERS

To investigate the involvement of the CTC circuit in the secondary generalization of limbic seizures at the cellular level, we performed in vivo single cell electrophysiologic recordings under neuroleptanesthesia.<sup>28</sup>

We first compared interictal neuronal firing patterns between unstimulated rats for each strain. The neuronal fir-

**Table 1. Interictal firing properties of TRN neurons**

Parameter analyzed	Unstimulated		Stimulated	
	NEC (n = 5 rats; 10 cells)	GAERS (n = 4; 7 cells)	NEC (n = 6; 10 cells)	GAERS (n = 5; 18 cells)
Firing frequency (Hz)	12.9 $\pm$ 2.6	7.3 $\pm$ 3.4	4.7 $\pm$ 1.5 <sup>‡</sup>	14.2 $\pm$ 2.0 <sup>***†</sup>
% Burst firing	11.8 $\pm$ 5.7	24.0 $\pm$ 7.4	33.4 $\pm$ 7.2 <sup>‡</sup>	8.7 $\pm$ 2.8 <sup>***†</sup>
Mean number of APs/burst	3.2 $\pm$ 0.3	5.2 $\pm$ 1.3	4.5 $\pm$ 0.5	3.0 $\pm$ 0.3 <sup>**</sup>
Maximum number of APs/burst	4.6 $\pm$ 0.5	6.6 $\pm$ 1.6	6.8 $\pm$ 0.8	4.5 $\pm$ 0.7 <sup>*</sup>
Intraburst firing frequency (Hz)	381.4 $\pm$ 25.1	383.9 $\pm$ 28	386.5 $\pm$ 22.6	316.8 $\pm$ 13.3 <sup>***†</sup>

AP, action potential.  
Data are expressed as mean  $\pm$  standard error of mean (SEM). Mann-Whitney *U* test was used for all statistical comparisons.  
\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, stimulated NEC versus stimulated GAERS. †*p* < 0.05, unstimulated NEC versus stimulated NEC. ‡*p* < 0.05, unstimulated GAERS versus stimulated GAERS.

**Table 2. Interictal firing properties of VPM neurons**

Parameters analyzed	Unstimulated		Stimulated	
	NEC (n = 5 rats; 8 cells)	GAERS (n = 5; 10 cells)	NEC (n = 9; 20 cells)	GAERS (n = 6; 11 cells)
Firing frequency (Hz)	3.8 ± 0.7	14.3 ± 3.4 <sup>####</sup>	5.9 ± 1.7	12.1 ± 2.4 <sup>**</sup>
% Burst firing	15.7 ± 6.6	4.3 ± 1.3	24.7 ± 4.9	1.0 ± 0.4 <sup>**</sup>
Mean number of APs/burst	2.7 ± 0.2	2.3 ± 0.1 <sup>#</sup>	2.9 ± 0.2	2.2 ± 0.2 <sup>**</sup>
Maximum number of APs/burst	3.9 ± 0.3	3.5 ± 0.4	4.2 ± 0.2	2.6 ± 0.3 <sup>**</sup>
Intraburst firing frequency (Hz)	296.5 ± 22.5	310.5 ± 12	384.5 ± 14.9 <sup>‡</sup>	285.9 ± 10.8 <sup>**</sup>

AP, action potential.  
Data are expressed as mean ± SEM. Mann–Whitney *U* test was used for all statistical comparisons.  
<sup>#</sup>*p* < 0.05, <sup>####</sup>*p* < 0.001, unstimulated NEC versus unstimulated GAERS. <sup>\*\*</sup>*p* < 0.01, <sup>\*\*\*</sup>*p* < 0.001, stimulated NEC versus stimulated GAERS. <sup>‡</sup>*p* < 0.01, unstimulated NEC versus stimulated NEC rats.

**Table 3. Interictal firing properties of CA3 pyramidal layer of hippocampal neurons**

Parameters analyzed	Unstimulated		Stimulated	
	NEC (n = 6 rats; 13 cells)	GAERS (n = 7; 10 cells)	NEC (n = 12; 24 cells)	GAERS (n = 9; 20 cells)
Firing frequency (Hz)	6.1 ± 0.7	8.3 ± 1.4	8.3 ± 1.3	8.8 ± 1.5
% Burst firing	2.1 ± 1.3	5.1 ± 3.9	6.3 ± 2.0	1.8 ± 1.0
Mean number of APs/burst	2.2 ± 0.1	2.1 ± 0.1	2.8 ± 0.2 <sup>‡</sup>	2.2 ± 0.1 <sup>*</sup>
Maximum number of APs/burst	2.6 ± 0.4	2.5 ± 0.2	4.4 ± 0.4 <sup>‡</sup>	2.5 ± 0.2 <sup>**</sup>
Intraburst firing frequency (Hz)	294 ± 15.3	322.9 ± 21.7	343.9 ± 12.4 <sup>‡</sup>	292.6 ± 12.8 <sup>*</sup>

AP, action potential.  
Data are expressed as mean ± SEM. Mann–Whitney *U* test was used for all statistical comparisons.  
<sup>\*</sup>*p* < 0.05, <sup>\*\*</sup>*p* < 0.01, stimulated NEC versus stimulated GAERS. <sup>‡</sup>*p* < 0.05, unstimulated NEC versus stimulated NEC rats.

**Table 4. Interictal firing properties of layers IV/V primary somatosensory cortex pyramidal neurons**

Parameters analyzed	Unstimulated		Stimulated	
	NEC (n = 4 rats; 7 cells)	GAERS (n = 6; 20 cells)	NEC (n = 11; 19 cells)	GAERS (n = 10; 30 cells)
Firing frequency (Hz)	3.5 ± 0.7	9.3 ± 1.5 <sup>#</sup>	5.9 ± 0.7	7.2 ± 1.1
% Burst firing	0.2 ± 0.2	1.8 ± 0.7	1.3 ± 0.9	2.9 ± 1.8
Mean number of APs/burst	2.2 ± 0.2	2.2 ± 0.1	2.0 ± 0.1	2.3 ± 0.2
Maximum number of APs/burst	2.2 ± 0.2	2.5 ± 0.2	2.3 ± 0.1	2.9 ± 0.5
Intraburst firing frequency (Hz)	290 ± 18.0	323.6 ± 11	280.1 ± 2.5	284.5 ± 5.9 <sup>†</sup>

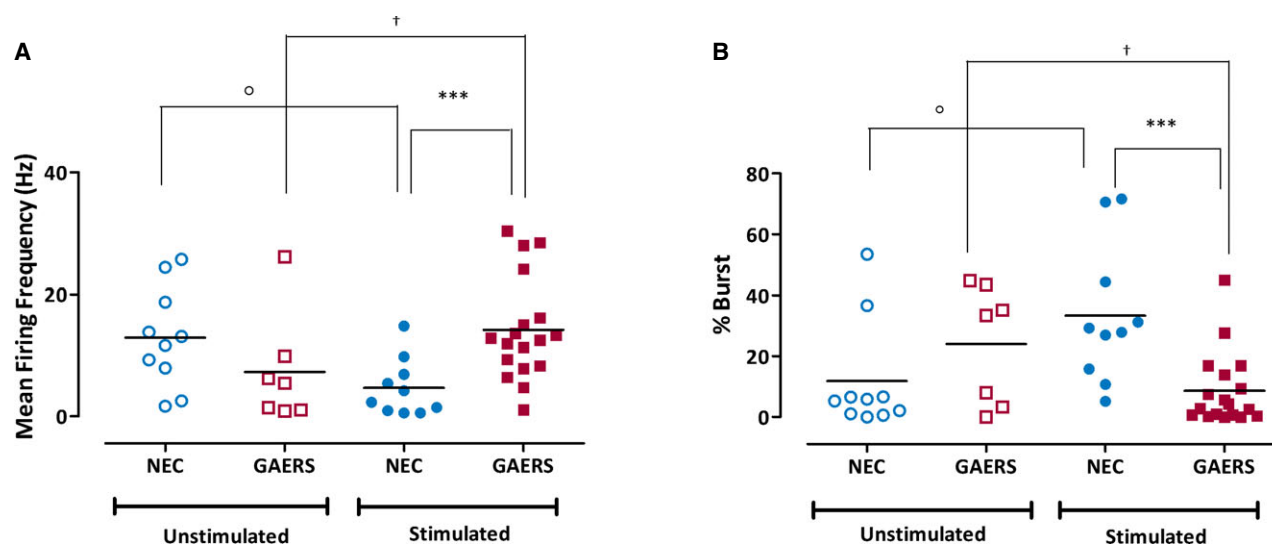
AP, action potential.  
Data are expressed as mean ± SEM. Mann–Whitney *U* test was used for all statistical comparisons.  
<sup>#</sup>*p* < 0.05, unstimulated NEC rats versus unstimulated GAERS <sup>†</sup>*p* < 0.05, unstimulated GAERS versus stimulated GAERS.

ing patterns recorded in CA3 pyramidal layer of the hippocampus and TRN were similar between unstimulated-NEC rats and unstimulated GAERS in all parameters examined (mean firing frequency, percentage burst firing, mean number of APs per burst, maximum number of AP per burst, and intraburst firing frequency) (Tables 1–4). However, in the VPM, and to a lesser extent in the somatosensory cortex, the spontaneous firing rate differed between the two strains. VPM neurons in unstimulated GAERS display significantly faster (14.3 ± 3.4 Hz) spontaneous firing rate than those in unstimulated-NEC rats (3.8 ± 0.7 Hz) (Table 2; *p* = 0.001). In VPM neurons, tonic firing frequency was also significantly higher (15.4 ± 4 Hz) in unstimulated GAERS compared to unstimulated-NEC rats (3.3 ± 1.3 Hz; *p* = 0.02), but no difference was observed

in burst firing frequency (Table S1). The interictal mean number of APs per burst was significantly lower in unstimulated GAERS (2.3 ± 0.1) compared to unstimulated NEC in VPM (2.7 ± 0.2) (Table 2; *p* = 0.05). Similar to VPM neurons, the layer IV/V somatosensory cortex pyramidal neurons of unstimulated GAERS (9.3 ± 1.5 Hz) showed significantly faster firing rates compared to unstimulated NEC (3.5 ± 0.7 Hz) (Table 4; *p* = 0.02).

#### Electrical amygdala-kindling stimulations result in a switch from tonic to burst firing of TRN neurons in NEC rats but not in GAERS

We next investigated the neuronal firing patterns after 30 electrical amygdala-kindling stimulations in GAERS and NEC rats. Kindling induced significant



**Figure 1.**

The interictal firing pattern of TRN neurons of NEC and GAERS groups. **(A)** The interictal firing frequency (Hz) showing decreases following kindling stimulations in NEC rats and increases in GAERS rats **(B)** the percentage of burst firing changes, which show decreases in GAERS following kindling stimulations and increases in NEC rats. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , show statistical difference between the stimulated NEC versus stimulated GAERS. ° $p < 0.05$ , unstimulated NEC versus stimulated NEC. † $p < 0.05$ , unstimulated GAERS versus stimulated GAERS (Mann-Whitney *U* test). Data were expressed as median  $\pm$  standard deviation (SD).

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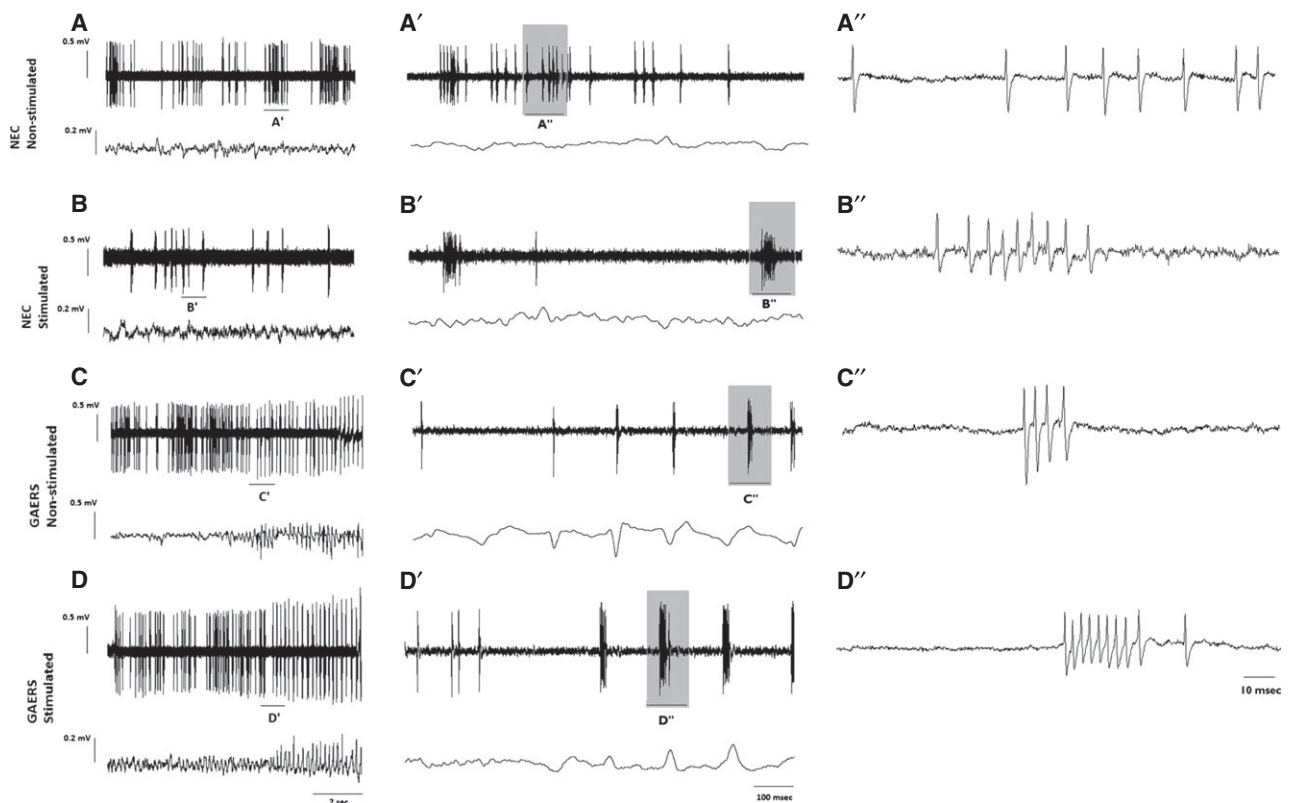
changes in neuronal firing patterns within the thalamus, most marked in the TRN, and to a lesser extent in the CA3 pyramidal layer of hippocampus in both GAERS and NECs.

In the TRN of NEC rats, following amygdala kindling the neuronal firing pattern displayed an overall lower firing frequency ( $p = 0.02$ ), with higher proportion of burst rather than tonic discharges ( $p = 0.02$ ) compared to unstimulated-NEC rats (Figs 1A,B and 2A,B; Table 1). When we examined the tonic and burst-firing rates separately in stimulated-NEC rats there was a strong trend of decrease in the tonic firing rate compared to unstimulated-NEC rats ( $3.6 \pm 1.2$  Hz vs.  $10.4 \pm 2.4$  Hz, respectively,  $p = 0.06$ ), but no significant change in the burst firing rate ( $1.1 \pm 0.4$  Hz vs.  $0.6 \pm 0.1$  Hz, respectively) (Table S1). This suggests that the decrease in overall firing frequency primarily reflected a switch in the predominant mode of firing from tonic to burst mode. In support of this, in NEC rats there was a significant negative relationship between percentage burst firing and mean firing frequency ( $R^2 = 0.89$ ). In stimulated GAERS that had been subjected to the same number of amygdala-kindling stimulations, neurons fired more frequently ( $p = 0.02$ ) with a significantly lower frequency of burst firing ( $p = 0.02$ ) and significantly lower intraburst frequency ( $p = 0.02$ ) compared to unstimulated GAERS (Figs 1A,B and 2C,D; Table 3). As in NEC rats, a significant relationship was present between percentage burst firing and mean firing frequency in GAERS ( $R^2 = 0.64$ ). In stimulated GAERS, tonic-firing frequency ( $13.9 \pm 2.6$  Hz) was significantly increased ( $p = 0.03$ ) compared to

unstimulated GAERS ( $3.2 \pm 1.4$  Hz); however, burst firing frequencies (stimulated:  $0.9 \pm 0.2$  Hz; unstimulated:  $0.9 \pm 0.4$  Hz) did not show a difference. When we compared stimulated NEC and stimulated GAERS, the tonic firing frequency in GAERS was significantly higher ( $p = 0.007$ ). No statistical difference was found in terms of burst frequency (Table S1). Firing properties of TRN neurons was also significantly different between the stimulated NEC rats and stimulated GAERS in all parameters analyzed (Fig. 1A,B; Table 1). The representative traces of TRN unit recordings from NEC rats and GAERS illustrate the effect of kindling stimulations on firing properties of TRN neurons in GAERS and NEC rats (Fig. 2A–D). Figure 2 clearly indicates the effect of kindling stimulation on the firing pattern of TRN neurons in both strains. After 30 kindling stimulations in NEC rats, firing of TRN neurons developed more burst firing and low frequency pattern with an enlarged single bursts (Fig. 2A''–D'').

#### Electrical amygdala-kindling stimulations altered firing properties of VPM neurons in NEC rats but not in GAERS

Within the VPM, amygdala kindling induced a significant increase in intraburst firing frequency of NEC rats ( $p = 0.01$ ), which was not observed GAERS. In the VPM, kindling stimulations did not alter the other firing properties examined in either strain compared to their unstimulated control groups (Table 2). Firing properties of VPM neurons differed significantly between the stimulated-NEC rats and stimulated GAERS in all parameters analyzed (Table 2).



**Figure 2.**

Representative extracellular recordings of TRN neurons in NEC rats and GAERS. Amygdala kindling led to a decrease in firing rate and an increase in percentage bursting of TRN neurons in NEC rats, with these changes not seen in stimulated GAERS rats. For each set of traces, upper trace is the unit activity of a single TRN neuron, and the second trace is EEG recorded from cortex. Traces were enlarged for higher magnification: **A'**, **B'**, **C'**, and **D'**, a 1 s period of traces labeled with **A**, **B**, **C**, and **D**. **A''**, **B''**, **C''**, and **D''**, a 100 msec period of traces labeled with **A'**, **B'**, **C'**, and **D'**. Rectangular gray areas show the enlarged 100 msec periods that indicate the features of the representative burst and isolated APs. **(A)** Record from a TRN neuron in a nonstimulated NEC rat that did not receive any kindling stimulations from amygdala. **(B)** Record from a TRN neuron in a stimulated NEC that received 30 kindling stimulations from amygdala. It is shown that stimulated-NEC rats developed the low frequency, burst firing pattern following kindling compared to nonstimulated NEC rats. **(C)** Record from a TRN neuron in a nonstimulated GAERS; the TRN firing pattern interictally was similar to nonstimulated NEC rat. The emergence of an SWD can be seen toward the end of the trace. **(D)** Record from a TRN neuron in a stimulated GAERS that showed resistance for developing low frequency bursting firing pattern.

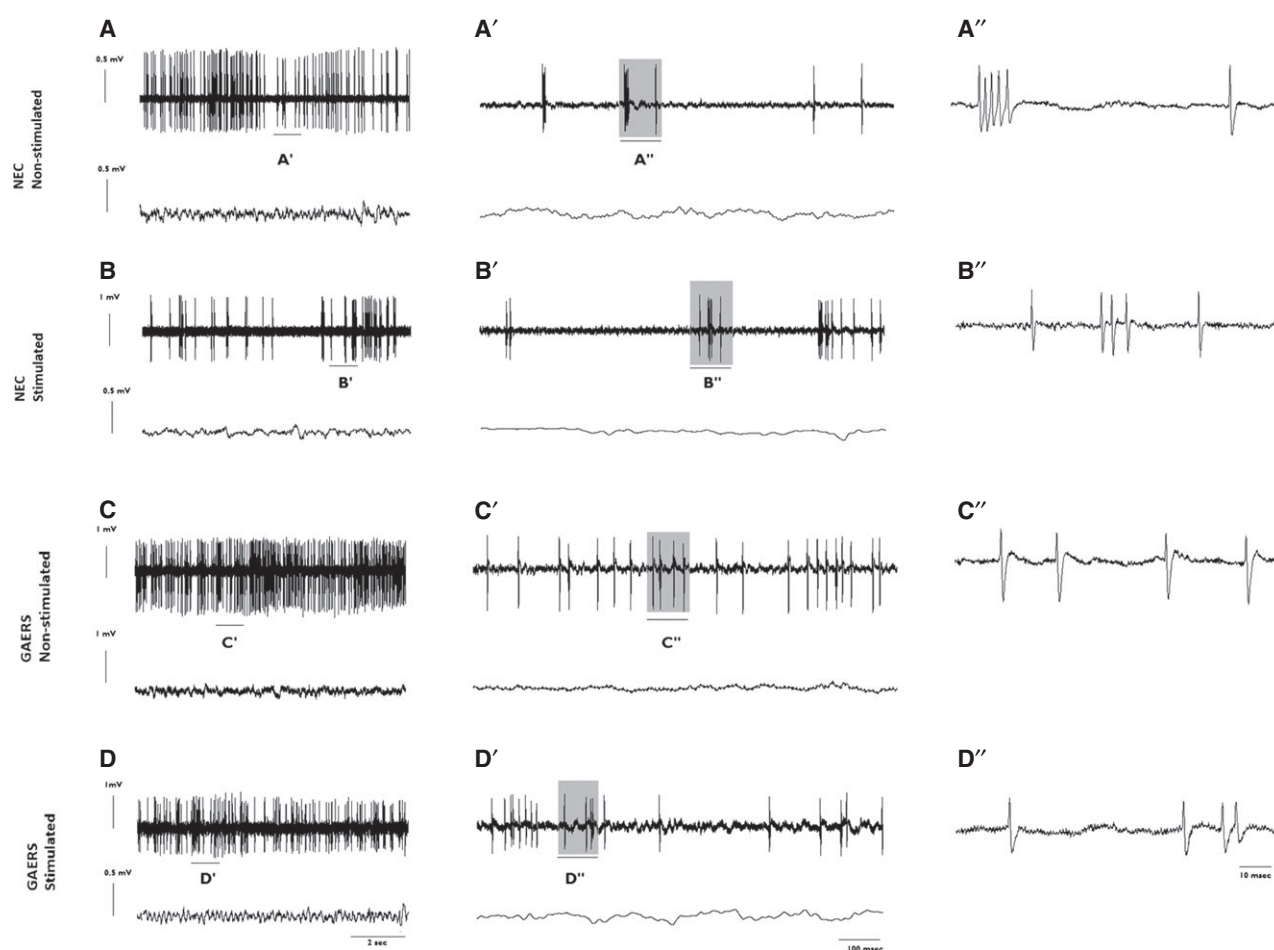
*Epilepsia* © ILAE

Tonic firing frequency in stimulated GAERS ( $16.1 \pm 5.2$  Hz) was significantly higher when compared to stimulated-NEC rats ( $5.1 \pm 1.7$  Hz;  $p = 0.01$ ); however, no difference was observed in burst firing frequency (Table S1). The representative traces of VPM unit recordings from NEC rats and GAERS illustrates the effect of kindling stimulations on firing properties of VPM neurons in GAERS and NEC rats (Fig. 3A–D). As shown on the trace, the firing frequency of VPM neurons in stimulated NEC rats (Fig. 3B,B') was significantly slower compared to that of stimulated GAERS (Fig. 3D,D').

### Electrical amygdala-kindling stimulations altered firing properties of hippocampal CA3 neurons of in NEC rats but not in GAERS

Electrical amygdala-kindling stimulations significantly altered the firing properties of CA3 hippocampal neurons of

NEC rats but not of GAERS. In the CA3 pyramidal layer of hippocampal neurons, elevated intraburst firing frequency with an increase in number and maximum number of APs per burst was observed in stimulated-NEC rats when compared to unstimulated-NEC rats. However in stimulated GAERS no significant difference was seen in any parameters compared to unstimulated GAERS (Fig. S3C,D, Table 3). Amygdala kindling significantly altered the firing properties of CA3 hippocampal neurons between the stimulated-NEC rats and stimulated GAERS (Table 3). Specifically, CA3 hippocampal neurons in stimulated-NEC rats had a higher intraburst frequency than in stimulated GAERS (Table 3;  $p = 0.01$ ). Representative traces of CA3 hippocampal units from NEC rats and GAERS shows that CA3 pyramidal neurons did not appear to undergo the firing-pattern alterations after kindling stimulation that were observed in thalamus (Fig. S3A–D).



**Figure 3.**

Representative extracellular recordings of VPM neurons in NEC rats and GAERS. NEC rats that had undergone amygdala-kindling stimulations showed a lower firing frequency of VPM neurons with an increased bursting pattern compared with stimulated GAERS. For each set of traces, upper trace is the unit activity of a single VPM neuron and the second trace is EEG recorded from cortex. Traces were enlarged for higher magnification: **A'**, **B'**, **C'**, and **D'**, a 1 s period of traces labeled with **A**, **B**, **C**, and **D**. **A''**, **B''**, **C''**, and **D''**, a 100 msec period of traces labeled with **A'**, **B'**, **C'**, and **D'**. Rectangular gray areas show the enlarged 100-msec periods and indicate the features of the representative burst and isolated APs. **(A)** Record from a VPM neuron in a nonstimulated NEC that did not receive any kindling stimulations from amygdala. **(B)** Record from a VPM neuron in a stimulated NEC, which received 30 kindling stimulations from amygdala. **(C)** Record from a VPM neuron in a nonstimulated GAERS. Higher firing rate can be observed compared to nonstimulated NEC. **(D)** Record from a VPM neuron in a stimulated GAERS.

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### Following electrical amygdala stimulations the intraburst firing frequency of layers IV/V of primary somatosensory cortex pyramidal neurons was reduced in GAERS but not in NEC rats

Kindling stimulations did not lead to any significant difference in firing patterns of layers IV/V of primary somatosensory cortex pyramidal neurons in NEC rats (Fig. S4). However, in GAERS following kindling the intraburst frequency of layers IV/V of primary somatosensory cortex pyramidal neurons was reduced when compared to unstimulated GAERS (Table 4;  $p = 0.02$ ). As clearly seen in Figure S4, layers IV/V of primary somatosensory cortex pyramidal neurons did not show a significant firing pattern alteration after kindling stimulations in either NEC rats or GAERS.

### Cross-correlation analysis demonstrated that kindling stimulations increased synchrony of simultaneous local field potentials between TRN and amygdala

The cross-correlation analysis on the local field potentials between amygdala and the TRN showed before kindling stimulations only modest synchronized activity between the local field potentials in the TRN and the amygdala in both the GAERS and NEC rats. The GAERS (prestimulation) showed some synchronous activity at SWDs of 5–6 Hz, which was not seen in the NEC rats in which the synchronized activity was in the 2–3 Hz range. After the kindling stimulation in the amygdala, the cross-correlation graph showed that there was a marked increase in synchrony between the two regions in both the GAERS and NECs at

limbic seizure frequency (2–3 Hz). Data are shown as Figure S5A,B.

## DISCUSSION

It has been postulated that the resistance of GAERS to the progression to the later stages of kindling, where convulsive seizures are manifest, may result from the effects of the preexisting SWDs in the CTC circuit, which inhibits the secondary generalization of limbic seizures.<sup>6,9,23,24</sup> The present study investigated the changes in single neuron firing patterns in vivo within the CTC circuitry that are associated with this kindling resistance in GAERS. The main findings from our study were the following: (1) In the NEC rats, amygdala kindling led to a decrease in firing rate and an increase in percentage bursting of TRN neurons, resembling synchronized activity, with less marked changes being seen in the VPM, CA3 pyramidal layer of hippocampus, and the deep layers of the somatosensory cortex; (2) in GAERS, the same number of amygdala stimulations failed to induce the changes in interictal neuronal firing patterns seen in TRN or VPM neurons in stimulated-NEC rats; (3) no changes in interictal firing patterns were seen in layers IV/V primary somatosensory cortex pyramidal neurons of unstimulated- and stimulated-NEC and GAERS in all parameters evaluated except intraburst firing frequency in GAERS; and (4) a higher intraburst frequency in CA3 hippocampal neurons of stimulated NEC was observed compared to that in stimulated GAERS.

A potential limitation of the study is that the recordings were acquired under fentanyl-haloperidol anesthesia. Ideally the observations would have been made in awake in vivo unrestrained recordings, but this would have been technically difficult. However, neuroleptanesthesia produces a state of brain activity wherein the background activity on EEG is similar to that of wakefulness and light slow wave sleep, and allows bilaterally synchronized SWDs to occur, closely resembling on the EEG that of unanesthetized conditions.<sup>28,32</sup>

### The role of TRN in secondary generalization of limbic seizures

A series of studies have demonstrated that the expression of SWDs in animal models of absence epilepsy results in resistance to the induction of experimental limbic epileptogenesis, in particular the secondary generalization of electrically kindled limbic seizures.<sup>7,24</sup> The circuitries involved in the generation of limbic and absence seizures were previously thought to be distinct: Absence seizures occur as a result of hypersynchronized oscillations within the CTC loop, whereas temporal lobe seizures are associated with pathologic and electrophysiologic changes in the limbic structures including the amygdala, hippocampus, and entorhinal cortex.<sup>33–35</sup> However, reciprocal connections

between the thalamus and the limbic structures suggest possible involvement of the thalamic regions in the propagation of temporal lobe seizure activity.<sup>36,37</sup>

Although a number of studies have indicated that the excitatory neural transmission from the thalamus may facilitate limbic seizures, the thalamic cellular mechanism associated with limbic seizure generalization is unclear.<sup>38,39</sup> Our findings indicate that the kindling-induced functional reorganization of the neural circuitry in NEC rats is associated with alterations in the neuronal firing patterns in the TRN, and to a lesser extent in the VPM. This presumably facilitates a secondary generalized spread of the seizure activity from the limbic system to widespread cortical and subcortical regions of both cerebral hemispheres. Our results indicate that the kindling resistance in GAERS is associated with the resistance in kindling-induced alteration in the firing pattern in the TRN neurons, suggesting that the TRN plays an important role in the secondary generalization of limbic seizure activity, a decrease in firing rate and an increase in percentage burst of the TRN neurons indicates that the activity of the local thalamic circuitry has been transformed from tonic to bursting state. In addition, these kindling-induced firing patterns are most prominently observed in the TRN and VPM, with only relatively mild changes seen in the hippocampus and the somatosensory cortex. These findings, which are consistent with those found in another study of neuronal firing patterns in kindled NEC rats, supports the hypothesis that the thalamus facilitates the relay and distribution of seizure activity to neocortical areas in the secondary generalization of limbic seizures.<sup>40</sup>

Possible underlying mechanisms for the observed descriptive findings of TRN induced by kindling in NEC rats need further experimental exploration. It has been shown that calcium currents are involved in kindling epileptogenesis.<sup>41</sup> Moreover, as several studies have shown the necessity of dendritic calcium spikes for burst generation, increased low threshold calcium currents could increase the bursting probability of TRN neurons in NEC rats.<sup>42</sup> Previous work by our group has shown that treating GAERS with the T-type calcium channel blocker, ethosuximide, overcomes their resistance to the progression of amygdala kindling.<sup>23,24</sup> However, whether this is a direct effect of the calcium channel blockage on limbic seizure propagation, or an indirect effect via the suppression of SWDs, is still uncertain.

### Thalamic resonance in limbic and absence seizures

The resistance to the convulsive stages of amygdala kindling in GAERS may represent a failure of the thalamic structures to resonate with the seizure activity occurring within the limbic structures, thereby inhibiting seizure propagation. This is reflected at the cellular level, as the TRN cells in GAERS were resistant to the kindling-induced switch (low frequency and high bursting state) observed in kindled NEC rats. Our results are consistent

with the concept that the thalamus is an important component of generalization of seizures from the limbic seizure circuitry. Experiments over recent decades, using lesion and pharmacologic studies, have demonstrated a modulatory role of the thalamus in limbic seizure development.<sup>38,43–46</sup> In this study, cross-correlation of field potential recordings shows that following amygdala-kindling stimulation, there is an increase in synchrony between the TRN and the amygdala in both the GAERS and NEC rats at the limbic seizure frequency (2–3 Hz) (Fig. S5). This indicates that in GAERS the TRN can engage in the rhythmic limbic seizure frequency from the amygdala, but appears not to be able to propagate this rhythm to other regions of the brain as occurs in the NEC rats.

However, the role of the TRN in the generalization of seizures is a relatively unexplored issue in epilepsy research. The results of the present study provide a better understanding of thalamocortical influence on seizure generalization in the kindling paradigm. There are numerous track-tracing studies demonstrating anatomic connections between the limbic system and the thalamus.<sup>37,47</sup> Furthermore, the thalamus has been used as a target for blocking seizure propagation. For example, stimulation of anterior nucleus of the thalamus influences the generalization of seizures, and GABA infusion in the dorsomedian nucleus of the thalamus elevates the generalized seizure threshold.<sup>48,49</sup> In addition, electrical stimulation of the TRN has been demonstrated to inhibit the generalization of seizures triggered by costimulation of the hippocampus, whereas stimulation in the mediodorsal thalamus showed no protection against amygdala kindling.<sup>19,50</sup> Although the exact role of TRN, VB, and midline thalamus in limbic seizure propagation remains to be completely elucidated, it is likely that engagement of thalamic neuronal firing with excitatory drives is required for both limbic and absence seizures to occur, depending on the pathologic state of the thalamus. In absence seizures, 5–9 Hz seizure precursor activities are observed in both GAERS and NEC rats that are generated in the somatosensory cortex.<sup>51</sup> SWD seizures occur as a result of pathologic hypersynchrony of the CTC system where the TRN and VB resonate in response to the cortical rhythm. A potential parallel between the seizure types would be that the generalization of an amygdaloid-kindled seizure also requires the resonance of the TRN and VB with the oscillatory excitatory input from limbic structures. The existing pathophysiological CTC network changes in GAERS—which result in a resonance frequency for the firing of the CTC structures, in particular the TRN and VB, at the frequency of the SWDs (5–8 Hz)—may inhibit the limbic structures from resonating at the lower oscillatory frequency of the limbic seizures. This would thereby impede the generalization of seizure discharges from the limbic structures, resulting in the resistance to the progression to the later stages of kindling in GAERS compared to NEC rats.

### The role of the cortex in the resistance of GAERS to the progression of limbic seizures

The cortex plays a critical role in convulsive seizures in kindled rats, and in human secondary generalized seizures.<sup>52,53</sup> During the convulsive stage of these seizures, widespread cortical areas, including motor and somatosensory cortex, show synchronized oscillatory firing engaging cortical spinal neurons to result in rhythmic bilateral muscle jerking. In this study we observed that in unstimulated GAERS, the baseline neuronal firing frequency in the deep cortical layers (V/VI) was higher than in unstimulated NEC rats (Table 4). In addition, stimulated GAERS developed reduced interburst frequency in the deep cortical layers compared to unstimulated GAERS. The frequency of the burst firing is known to influence the strength of the postsynaptic response, with reduced intraburst frequencies resulting in a larger postsynaptic response.<sup>54,55</sup> However, whether the changes in cortical neuronal firing play a role in the resistance to progression of amygdala kindling seen in GAERS requires further research.

## CONCLUSIONS

By characterizing the neuronal firing patterns in the CTC circuitry, this study demonstrated that in the NEC rats, the kindling stimulations lead to an alteration of the firing patterns of the TRN and VPM neurons into low frequency and highly bursting pattern, whereas the GAERS are resistant to kindling and the associated firing pattern changes in these structures. These findings suggest a role of the TRN and VPM structures in the resistance to the progression of amygdala-kindling induced convulsive seizures and with potential implications for the mechanisms of secondary generalization of temporal lobe seizures in humans.

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## DISCLOSURE

None of the authors has any conflict of interest to disclose. 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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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Data S1.** The preparation of in vivo extracellular recordings, juxtacellular labeling, and histology.

**Table S1.** Tonic and burst firing frequencies of TRN and VPM neurons.

**Figure S1.** Location of glass electrodes and juxtacellularly labeled VPM and TRN neurons.

**Figure S2.** Seizure progression over 30 amygdala stimulations in NEC rats and GAERS with representative EEG traces showing afterdischarges.

**Figure S3.** Representative extracellular recordings of CA3 pyramidal layer of hippocampal neurons in NEC rats and GAERS.

**Figure S4.** Representative extracellular recordings of layers IV/V primary somatosensory cortex pyramidal neurons in NEC rats and GAERS.

**Figure S5.** Cross-correlation of simultaneous local field potentials between the amygdala and the TRN in NEC rats and GAERS.