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Ca_v3.2 T-Type calcium channel mutation influences kindling-induced thalamic neuronal firing patterns in Genetic Absence Epilepsy Rats from Strasbourg

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Abbreviated title: Cav3.2 T-Type calcium channel mutation vs. kindling in GAERS

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Abstract

Objective: Recent data implicate that amygdala kindling leads significant changes in interictal neuronal firing patterns of thalamic reticular nucleus (TRN) neurons by decreasing the spontaneous firing rate and increasing burst firing in non-epileptic control (NEC) rats. Genetic Absence Epilepsy Rats from Strasbourg (GAERS) were resistant to these-kindling induced firing changes in TRN neurons, and are also resistant to the progression of kindling. We investigated whether a homozygous, missense, single nucleotide mutation (R1584P) in the $Ca_v3.2$ T-type Ca^{2+} channel gene, which has been correlated with the expression of absence seizures in GAERS, influenced kindling progression and TRN firing patterns.

Methods: Double crossed (GAERS vs.NEC) (F2) rats that were homozygous for the $Ca_v3.2$ mutation (PP), and those negative for the mutation (RR) were implanted, with a stimulating electrode in the amygdala. Rats received a total of 30 kindling stimulations at their afterdischarge threshold (ADT) current twice daily and kindling progression was evaluated. Thereafter the extracellular neuronal activity of TRN neurons were recorded in-vivo under neuroleptanesthesia to investigate the influence of $Ca_v3.2$ mutation on TRN firing patterns.

Results: We found that the R1584P mutation did not affect kindling progression in F2 crosses ($p=0.78$). However, it influenced kindling-induced neuronal firing of TRN neurons. After 30 stimulations, RR rats exhibited a lower firing rate and a higher percentage of burst firing compared to PP rats. The decrease in firing frequency was correlated with the increase in the amount of burst firing in RR rats ($R^2=0.497$).

Significance: Our findings suggest that mutation in $Ca_v3.2$ T-type Ca^{2+} channels may play a role in the resistance to kindling-induce changes in TRN neurons to a low frequency and high percentage bursting pattern seen in association with the convulsive stages of amygdala kindling, but is not in itself enough to explain the resistance to kindling progression observed in GAERS.

Key points:

- TRN neurons in double-crossed (F2) GAERS vs. NEC rats null for the mutation (RR) exhibited a lower firing rate and a higher percentage of burst firing compared to the rats homozygous for the mutation (PP) rats post-amygdala kindling.

- The decrease in TRN total firing frequency was correlated with the increase in the amount of burst firing in RR rats
- The mutations in $\text{Ca}_v3.2$ T-type Ca^{2+} channels influenced kindling-induced neuronal firing of TRN neurons however did not affect the kindling progression in GAERS.

Introduction

Repeated electrical amygdala kindling stimulations decrease spontaneous firing rate and enhance burst firing activity of thalamic reticular nucleus (TRN) neurons in the non-epileptic control (NEC) rat strain¹. Associated with the kindling resistance observed in Genetic Absence Epilepsy Rats from Strasbourg (GAERS), TRN neurons showed resistance to the kindling-induced switch to a low frequency and high bursting state that is observed in kindled NEC rats.^{1,2} Outputs from the TRN appear to be critical in the resistance to the generalization of limbic seizures, as well as the synchronization of SWDs in GAERS³. Rhythmic burst firing mediated by low-voltage-activated T-type channels in TRN and thalamocortical neurons are required for the generation of thalamocortical oscillations and dysfunction of these channels, particularly the $\text{Ca}_v3.2$ (*Cacna1h*) subtype, has been implicated in the pathogenesis of absence epilepsy.^{4,5,6,7}

In GAERS, $\text{Ca}_v3.2$ mRNA expression⁵ and T-type Ca^{2+} currents⁴ have been found to be elevated in the TRN, and a gain of function mutation was identified in the $\text{Ca}_v3.2$ channel which is prominently expressed in the TRN⁷. Our previous work has shown that GAERS are resistant to kindling-induced neuronal firing alterations in TRN¹. We therefore hypothesised the presence of the gain-of-function GAERS R1584P $\text{Ca}_v3.2$ mutation plays an important role in this resistance to kindling-induced neuronal firing changes in the TRN, and to the resistance to the progression of kindling that these rats display. We tested this in a F2 generation of double crossed GAERS vs. their related non-epileptic control (NEC) strain that were homozygous for the R1584P $\text{Ca}_v3.2$ mutation vs. those negative for the mutation. NEC rats were derived from the same original colony as the GAERS, but have been selectively inbred so as to not express SWDs.^{8,9}

Materials and Methods

Animals

Adult (4-5 months old), male, weighed 250-350 g F2 offspring of double cross matings of GAERS x NEC rats (n=47) were used. Among 47 F2 animals, 24 rats were homozygous (PP) and 23 rats were null (RR) for the R1584P mutation. Due to the technical difficulties such as loss of headcaps from substantial number of animals as only half of the skull was secured with dental cement, the final number of animals that underwent all experimental procedures were n=5 for PP and n=9 for RR rats. Animals were maintained under standard laboratory conditions on a 12/12-h light/dark cycle, with ad libitum access to food and water. The study was approved by the Animal Ethics Committee of the Royal Melbourne Hospital, the University of Melbourne (Ethics number: 0706287) and conformed to National Health and Medical Research Council Guidelines for the ethical use of animals in scientific research. All efforts were made to minimize distress and the number of animals necessary to produce reliable data.

Breeding of the F2

The F2 generation was produced as previously described⁷; GAERS, which are homozygous (PP) for the R1584P $\text{Ca}_v3.2$ mutation, were crossed with NEC rats (null (RR) for the R1584P $\text{Ca}_v3.2$ mutation) to produce an F1 generation all of which are heterozygous (RP) for the mutation (Figure 1a). Two F1 generation rats (RP) were then crossed to produce an F2 generation. F2 rats were genotyped to identify those that were either homozygous (PP) or null

(RR) for the R1584P mutation as described before.⁷ The experimenters were blinded to the genotype of the rats until the completion of the experiments.

Surgery –~~electrode~~ implantation

F2 rats (RR and PP) were anesthetized with ketamine; (100 mg/kg; i.p.) plus xylazine (10 mg/kg; i.p.) and placed into a stereotaxic frame. A single midline incision was made on the scalp, and a bipolar stimulating electrode stereotaxically implanted in the left basolateral nucleus of the amygdala (BLA) (coordinates: 2.6 mm posterior, 4.8 mm lateral to bregma, 8.5 mm ventral from the surface of the skull).¹⁰ Holes were drilled on the skull and recording electrodes screwed over the left frontal (2 mm anterior, 3.5 mm lateral to bregma) and left occipital cortex (6 mm posterior, 4 mm lateral to bregma). A ground electrode was placed over the cerebellum. Recording electrodes comprised of a 1.3 mm male connector soldered onto a nickel alloy jeweler screw. All electrodes were fixed in position by applying dental cement around and only over the left hemisphere of the skull to leave the bregma and right hemisphere intact for electrophysiological recordings. The incision was then sutured. Rats were allowed a one-week recovery period before the start of the kindling procedure.

EEG Recordings and Analysis

Seven days after electrode implantation, prior to commencing the kindling stimulations, 24-hour EEG recordings were performed on all F2 rats using Compumedics™ EEG acquisition system (Melbourne, Australia). Animals were connected to an EEG board and remain in their home cages throughout the recording with food and water ad libitum. SWDs were detected and quantified using SWCfinder v5 (P. L. C. van den Broek; Netherlands). The standard criteria for SWD detection were used, i.e., an SWD episode of amplitude more than 3 times baseline, a frequency of 7-12 Hz and duration of longer than 0.5 s.^{11,12} From this, the total time in seizure over the 24 h recordings, individual seizure frequency and duration was determined.

Genomic DNA extraction and genotyping PCR

Genomic DNA was extracted from tail tips using the Promega Wizard Genomic DNA extraction kit, and genotyping PCR was performed using primers designed to amplify exon 24 (193 bp). Each 20 µl of PCR reaction contained the following: 1x Taq DNA polymerase buffer, 2.5 U of Taq DNA polymerase, 250 µM dNTPs, 500 nM forward and

reverse primers, and 25 ng of genomic DNA (Table 1) to confirm the correct size band, 5 μ l of PCR reactions were run on a 2% agarose gel with molecular weight markers, and gels were stained with GelRed DNA stain (Jomar) and visualized under UV light. PCR reactions were cleaned up using the Promega PCR clean-up kit, and purified PCR products were sent to the Australian Genome Research Facility (Melbourne, Australia) for sequencing (for primer sequences, see Table 1). Sequence analysis was done using Sequence Scanner version 1.0 (Applied Biosystems).

Electrical Amygdala Kindling

Electrical stimulations were delivered from an isolated constant current stimulator applied through the bipolar electrode (monophasic square-wave current; frequency = 80 Hz; duration = 2 s; pulse width = 1 ms) to BLA. Rats received a total of 30 stimulations at their afterdischarge threshold (ADT) current intensity twice daily, with an inter-stimulation interval of at least 4 h. Behavioral manifestations of the seizures induced by the electrical stimulations were classified according to the stages described by Racine: Class I- facial clonus; Class II- head nodding; Class III- contralateral forelimb clonus; Class IV-bilateral forelimb clonus and rearing; Class V- rearing and falling.¹³

Surgery – Electrophysiology

In vivo extracellular electrophysiology recordings of single neuronal firing patterns in the TRN, were performed according to the method described by Pinault D.¹⁴ One hour after the 30th kindling stimulation rats were deeply anesthetized with injection of ketamine (100 mg/kg; i.p.) plus xylazine (10 mg/kg; i.p.). Rats underwent tracheotomy to facilitate artificial ventilation in the pressure mode (8-12 cm H₂O; 60-65 breaths per minute (min)), and catheterization of the penile vein to facilitate intravenous (i.v.) neuroleptanesthesia. The neuroleptanesthesia was initiated before the end of the general ketamine-xylazine anaesthesia, then maintained by an i.v. injection of a mixture containing d-tubocurarine chloride, fentanyl, haldol and glucose.¹⁴ Rats were placed in a stereotaxic frame without removing previously implanted socket for EEG recordings then craniotomies and duratomies were made on the right hemisphere vertically above the TRN. The main physiological parameters (heart rate: 300-350 beats/min; rectal temperature: 37°C; breath rate: about 60 breaths/min) and the surface EEG continuously monitored throughout the experiment to ensure the stability of the anesthesia.

In vivo extracellular recordings

Starting at least 1 h after completion of all surgical procedures, the interictal patterns of single neurons in TRN were examined by in vivo extracellular single-unit recordings by using micropipettes prepared from 1.5 mm glass capillaries filled with a 1.5% solution containing N-(2-aminoethyl) biotinamide hydrochloride dissolved in 1 M CH₃COOK. Additionally, EEG recording from cortex and BLA were also performed. At the end of the recording sessions, neurobiotin was applied to the last pair of recorded neurons using single-cell labeling methods to identify the location and anatomy of the recorded cell.

Electrophysiological recordings and Analysis

The extracellular neuronal activity was recorded, and recordings were analyzed with the pClamp 10. Signals were digitized at a sampling rate of 20 kHz per channel, using pCLAMP 10. Data were processed with band passes of 0.1-1200 Hz for the EEG and 0-6000 Hz for cellular activity. Neuronal firing patterns were recorded for 15 min when the EEG was at a desynchronized background of <0.2 mV. All recorded neurons exhibit action potentials (APs) that can occur as either single APs or as bursts of AP. A burst is 2 or more APs firing within 6 ms of each other, measured from the onset of each AP. Recordings were divided into 3 segments, 60 s sample were selected from each of the segments for the analysis of neuronal firing patterns. Because many of the F2 rats experience absence seizures, the selection of 60 sec samples were screened to ensure they contained no seizure activity, and the firings patterns during these samples therefore represent interictal activity. 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 and averaged for each cell.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 5. Data were presented as mean $\pm$ standard deviations (SD). The Mann-Whitney *U* test was used for comparison of non-normally distributed continuous variables: ADTs, absence seizure frequency and duration, percentage of time in seizure activity and neuronal firing patterns between groups. Two-way ANOVA followed by the post hoc Bonferroni test was used to compare the kindling rate and afterdischarge duration between the groups. Correlations between the firing frequency vs. %

burst firing and kindling stage achieved vs % burst firing were tested using Pearson's correlation analysis. p values of < 0.05 were considered statistically significant.

Results

The R1584P Cav3.2 mutation is associated with the expression of absence seizures in F2 rats

Firstly, in order to confirm that the R1584P Cav_v3.2 mutation is associated with the expression of absence seizures in F2 rats, a 24-hour EEG were performed and used for seizure quantification. All F2 animals that possessed two copies of the mutation (PP, $n=23$) expressed SWDs. In rats null for the mutation (RR rats), 58% (14 of 24 rats) expressed SWD while the others showed no detectable seizure activity. In addition, rats homozygous for the mutation spent more time in seizure activity than those null for the mutation (PP vs. RR; $1.69 \pm 0.35\%$, $n=23$, vs. $0.55 \pm 0.24\%$, $n=24$; $p=0.015$) (Fig 1a). This was associated with in increased seizure frequency in PP rats (8.57 ± 1.44 seizure/hour, $n=23$; vs. 2.06 ± 0.75 , $n=24$; $p=0.0002$) (Fig 1b). In the rats that express SWDs, the percentage time in seizures were not different between the groups (PP vs. RR, $1.76 \pm 0.36\%$, $n=23$; vs. $1.06 \pm 0.38\%$, $n=14$; $p=0.19$). The length of individual seizures was not significantly different between the genotypes (PP vs. RR; 4.27 ± 0.60 s, $n=23$; vs 3.72 ± 0.65 , $n=14$; $p= 0.56$) (Fig 1c). Using F2 generation crosses between GAERS and NEC rats, our current data confirmed that this mutation is linked with absence seizure expression, reflected as an increase incidence and frequency of individual SWDs, consistent with the findings in our previous study.⁷

The progression of kindling is not influenced by the R1584P Cav_v3.2 mutation

One day after the EEG recordings, rats that homozygous or the mutation (PP, $n=5$) and rats null for the mutation (RR, $n=9$) those showed no seizure activity were administered 30 amygdala stimulations. Of the rats that were homozygous for the mutation (PP), 3 out of 5 (60%) progressed to Class IV-V seizures and 2 out of 5 (40%) did not progress beyond Class II seizures. Of the rats that did not carry the mutation (RR), 7 out of 9 (78%) reached Class IV-V seizures, 2 out of 9 (22%) reached Class III seizures. In RR rats, the amygdala kindling stage achieved correlated negatively with the percentage of burst firing of TRN neurons (Pearson r value= -0.82 , $p=0.006$) (Fig 3g). The rate of behavioral seizure progression and afterdischarge duration was not different between the groups (Fig 2a-b; $p>0.05$). The mean ADT also was not different between PP and RR rats (Fig 2c; 177 ± 108.8 μ A, vs. 190 ± 102.7 μ A, $p>0.05$).

The presence of the R1584P Ca_v3.2 mutation is associated with resistance to kindling induced changes in the TRN neuronal firing patterns

In a previous study, we demonstrated that following kindling, the most significant changes in interictal neuronal firing patterns observed in TRN which is the thalamic structure that has the highest expression of Ca_v3.2.⁵ Here we tested whether the F2 generation double crossed GAERS vs. NEC rats that were homozygous for the R1584P Ca_v3.2 mutation would also show resistance to the kindling induced changes in TRN interictal firing patterns. Consistent with this hypothesis, recordings of the TRN neurons in F2 animals who had received 30 amygdala stimulations, showed that the rats null for the mutation (RR) (9 rats; 36 cells) exhibited a lower firing rate and a higher percentage of burst firing interictally compared to the rats homozygous for the mutation (PP) rats (5 rats; 15 cells) (Fig 3a-e). Single TRN neurons in RR rats were markedly less active interictally (4.92 ± 5.08 vs. 12.26 ± 10.69 Hz, $p < 0.01$) with an effect driven by an increase in burst firing (23.19 ± 16.54 vs. 13.55 ± 15.41 Hz, $p < 0.05$). This was a very similar pattern to that previously observed in NEC rats who had undergone the same number of amygdala stimulations. As expected, a decrease in firing frequency was correlated with an increase in the amount of burst firing ($R^2 = 0.497$, Fig 2f). The number of APs per burst, intraburst frequency and the maximum number of APs per burst were not different between the two genotype groups (Fig 3c).

Discussion

The present study investigated the effect of the GAERS R1584P Ca_v3.2 gain of function mutation on kindling progression and kindling-induced neuronal firing changes in the TRN in double crossed F2 (GAERS vs. NEC) rats. F2 rats homozygous for the R1584P Ca_v3.2 mutation (PP) displayed resistance to the changes in TRN firing pattern induced by amygdala kindling stimulations, similar to that seen in GAERS, but showed no resistance to the kindling progression.

GAERS have been shown previously to have a resistance to the progression of amygdala kindling¹, but the mechanisms for this are uncertain. The single nucleotide mutation (R1584P) in the T-type calcium channel Ca_v3.2 gene has been shown by our group to influence the absence seizure expression in GAERS.^{7,15} In line with the previous findings, here we also demonstrated that PP rats spent more time in seizure activity compared to RR rats those are null for the mutation (Fig 1b). This current study was designed to investigate whether this mutation also played a mechanistic role in the resistance to kindling progression phenotype

seen in GAERS. Although the F2 rats did not show significant differences in the progression of kindling, we did find that at the single neuronal level the F2 rats homozygous for the R1584P $\text{Ca}_v3.2$ mutation (PP) showed a resistance to developing the low frequency burst firing pattern that is associated with kindling progression.¹⁶ In line with our findings, the gain-function mutations in $\text{Ca}_v3.2$ T-type Ca^{2+} channel has been shown to increase seizure susceptibility of cultured neurons by increasing the spontaneous firing rate.¹⁷ We also found that the kindling stage achieved negatively correlated with the percentage of burst firing of TRN neurons in RR rats (Fig 3g). We previously have shown that TRN neurons in GAERS has higher percentage of burst firing compared to NEC rats.¹ Moreover, the amygdala kindling rate correlated negatively with the intensity of SWDs.¹⁸ These new finding provide more supportive data for the hypothesis that the higher burst firing rate of TRN neurons contributes to resistance the progression of amygdala kindling. Further supporting this, the TRN has been directly implicated in the generalisation of limbic seizures: simultaneous electrical stimulation of the TRN slows kindling progression along with the severity of hippocampal kindling seizures.³

The $\text{Ca}_v3.2$ mutation in the GAERS leading to an increased $\text{Ca}_v3.2$ channel expression and T-type currents.¹⁹ The elevated T-type Ca^{2+} conductance in GAERS is thought to play an important role in the transformation between tonic and burst firing patterns in the TRN, leading to generation of oscillatory thalamo-cortical activity that characterize absence seizures.²⁰ The results of this study suggest that the R1584P $\text{Ca}_v3.2$ mutation in these rats plays a role in preventing the transformation of the TRN cells to a low frequency and high percentage bursting pattern seen in association with the later, convulsive stages, of amygdala kindling. Upregulation of $\text{Ca}_v3.2$ channels after status epilepticus produced an increase in T-type Ca^{2+} channel currents and was associated burst firing in wild type mice, but not in $\text{Ca}_v3.2$ knock-out mice.²¹ Selective blockage of T-type Ca^{2+} channels delayed seizure progression in the amygdala kindling rat model,²² and significantly inhibits absence seizures in GAERS.²³

In addition to alterations in $\text{Ca}_v3.2$ channels, changes in other Ca^{2+} channels have been described in GAERS rats and the related WAG/Rij model. In WAG/Rij rats increased expression of $\text{Ca}_v2.1$ (P/Q type high voltage activated) channels in the TRN was described,²⁴ and this is correlated with an increase of the T-type Ca^{2+} current and enhancement of SWD activity.²⁵ Moreover, the increased L-type Ca^{2+} current with an upregulated $\text{Ca}_v1.3$ expression in WAG/Rij rats, may contribute to increased thalamic burst firing and thereby the amygdaloid seizure progression.²⁶

Channelopathies in several ion channels have been identified as possible pathophysiological mechanisms of absence seizures in both humans and animal models.^{27,28} Among these different voltage-gated ion channels, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and the mediated *I_h* currents are proposed to contribute to network excitability and seizure expression in genetic models of absence epilepsy.^{29,30,31} Moreover, a reduction in HCN2 mRNA levels was also observed in fully kindled animals in the CA3 region.³² HCN channels that contributes to resting membrane potential and generation of burst firing in thalamocortical neurons with T-type Ca²⁺ channels, might be potentially involved in the seizure progression in amygdala kindling model – which is likely multifactoral.

Together, the data from this study establish that while Ca_v3.2 channels influence seizure progression in amygdala kindling model, the R1584P Ca_v3.2 mutation by itself is not enough to explain the mechanism by which GAERS are resistant to the progression of kindling.

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Disclosure of conflict of interest

None of the authors has any conflicts of interest.

Ethical publication statement

We confirm that we have read the Journal's position on issue involved in ethical publication and affirm that this report is consistent with those guidelines.

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Tables:

Table 1. Primer sequences for amplification of Ca_v3.2 exon 24 and for sequencing

	Forward primer	Reverse primer
PCR amplification of exon 24	5`-gaaccacaaccctggatgc-3`	5`-cctgcgcctcctctccag-3`
Sequencing primers	5`-cctggatgctgctctacttca-3`	5`-aagacgaagacgatggtaaaga-3`

Figure Legends

Figure 1. Absence seizure expression in F2 generation double crossed GAERS x NEC rats. *a*, The production of F2 generation. The two steps for producing double cross matings required for this study is shown. First step is the crossing GAERS rats (homozygous or PP for the R1584P Cav3.2 mutation) with non-epileptic control (NEC) rats (null or RR for the R1584P Cav3.2 mutation) to produce an F1 generation, all of which should be heterozygous (RP) for the mutation. Second step is the mating two F1 (RP) generation rats to produce an F2 generation. On average, 25% of the F2 progeny would be expected to be homozygous (PP) for the mutation, 50% heterozygous for the mutation (RP), and 25% null (RR) or not carrying the mutation at all. The R1584P Cav3.2 mutation is associated with the expression of absence seizures in F2 rats. *b*, Percentage of recording time spent in seizure activity. Animals homozygous for the mutation spend more time in seizure activity than animals null for the mutation (* $p < 0.05$, Mann Whitney U test), *c*, Seizure frequency expressed as number of

seizures per hour ($***p<0.001$, Mann Whitney U test). **d**, Individual seizure duration ($p>0.05$, Mann Whitney U test). Data expressed as mean $\pm$ SD.

Figure 2. Seizure progression over 30 amygdala stimulation in rats homozygous (PP) and null (RR) for the R1584P $Ca_v3.2$ mutation. **a**, Seizure stages. No significant difference was observed between the groups ($p>0.05$, Two-way repeated measures ANOVA). **b**, Afterdischarge duration from amygdala ($p>0.05$, Two-way repeated measures ANOVA). **bl**, Afterdischarge threshold. No difference was observed between the two genotypes (Mann Whitney U test, $p>0.05$). Data expressed as mean $\pm$ SD.

Figure 3. Firing pattern of TRN neurons of rats homozygous (PP) and null (RR) for the R1584P $Ca_v3.2$ mutation after 30 amygdala kindling stimulations. High-pass filtered traces showing typical isolated action potentials of TRN neurons of RR rats (top) **a**, and PP rats (bottom) **b**. The red asterisk indicates the bursts that are enlarged on the right. **c**, Table showing no significant difference in the number of APs per burst, the intraburst frequency and, the maximum number of APs per burst between the two genotypes. **d**, the interictal mean firing frequency (Hz). The rats homozygous (PP) for mutation show higher firing frequency than rats null for the mutation (RR) ($**p=0.0063$) (d-e). **e**, percentage burst firing. RR rats exhibit a significantly higher percentage of burst firing compared to the PP rats ($*p=0.0254$). **f**, a decrease in firing frequency is correlated with an increase in the amount of burst firing ($R^2=0.497$). **g**, Negative correlation between the kindling stage achieved and the percentage of burst firing of TRN neurons in RR rats ($R^2=0.682$, $n=9$). Data are expressed as mean $\pm$ SD.

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