

Ca_v3.2 drives sustained burst-firing, which is critical for absence seizure propagation in reticular thalamic neurons

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Summary

Objective: Genetic alterations have been identified in the *CACNA1H* gene, encoding the Ca_v3.2 T-type calcium channel in patients with absence epilepsy, yet the precise mechanisms relating to seizure propagation and spike-wave-discharge (SWD) pacemaking remain unknown. Neurons of the thalamic reticular nucleus (TRN) express high levels of Ca_v3.2 calcium channels, and we investigated whether a gain-of-function mutation in the *Cacna1h* gene in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) contributes to seizure propagation and pacemaking in the TRN.

Methods: Pathophysiological contributions of Ca_v3.2 calcium channels to burst firing and absence seizures were assessed in vitro using acute brain slice electrophysiology and quantitative real-time polymerase chain reaction (PCR) and in vivo using free-moving electrocorticography recordings.

Results: TRN neurons from GAERS display sustained oscillatory burst-firing that is both age- and frequency-dependent, occurring only in the frequencies overlapping with GAERS SWDs and correlating with the expression of a Ca_v3.2 mutation-sensitive splice variant. In vivo knock-down of Ca_v3.2 using direct thalamic injection of lipid nanoparticles containing Ca_v3.2 dicer small interfering (Dsi) RNA normalized TRN burst-firing, and in free-moving GAERS significantly shortened seizures.

Significance: This supports a role for TRN Ca_v3.2 T-type channels in propagating thalamocortical network seizures and setting the pacemaking frequency of SWDs.

KEY WORDS

absence epilepsy, low threshold spike, thalamocortical, T-type calcium channel

Cain and Tyson equally contributed to this work.

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1 | INTRODUCTION

Burst-firing of thalamic neurons is a characteristic cellular electrophysiological feature accompanying the spike-and-wave discharges (SWDs) seen on electroencephalography (EEG) recordings during absence seizures, and can also be observed in other generalized and focal epilepsies.¹ Thalamic neurons display similar firing patterns during the physiological thalamocortical oscillatory rhythms observed in non-rapid eye movement (REM) sleep, thus burst-firing per se is not a pathophysiological marker.^{2,3} However, during absence seizures, there is an inappropriate switching during wakefulness of thalamic neuronal firing from tonic to oscillatory burst-firing, and that is believed to play a key role in the impairment of consciousness during seizures by interrupting the normal relay of incoming sensory information.^{4,5} The ability of the thalamus to generate intrinsic oscillations, and the correlation between burst activity in thalamic reticular nucleus (TRN) neurons with spikes in SWDs suggest that the TRN may act as a thalamocortical pacemaker, and is therefore a strong candidate for initiating and/or propagating absence seizures.^{2,6,7} In rat models of absence epilepsy, a region of the primary somatosensory (S1Cx) and S2/insular cortex appears to be the focal point of seizure generation.^{8,9}

T-type calcium channels underlie the low threshold calcium spike (LTS) required to generate neuronal burst-firing in TRN and thalamocortical neurons. The magnitude of the LTS is directly related to the number of action potentials per burst in TRN, but not in cortex-projecting thalamocortical neurons such as those of the ventrobasal (VB) complex.^{2,10–12} Increased T-type messenger RNA (mRNA) expression and whole cell currents have been observed in TRN and thalamocortical neurons in genetic rat models of generalized epilepsy.^{13–15}

We reported previously that the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) model of genetic generalized epilepsy possess an arginine to proline missense (R1584P) mutation in the *Cacnalh* gene encoding the Ca_v3.2 T-type calcium channel.¹⁶ The point mutation co-segregates with SWD expression when outcrossed with Non-Epileptic Control (NEC) rats, whereby outcrossed offspring homozygous for the R1584P mutation display significantly enhanced seizure activity compared to offspring null for the mutation. Studies in exogenous expression systems indicate that mutation induces a gain-of-function phenotype in Ca_v3.2 channels, selectively in splice variants containing exon 25 (Ca_v3.2(+25)) found downstream of the mutation. The R1584P mutation increases the rate at which Ca_v3.2(+25) variant channels recover from inactivation and further results in increased calcium charge transference during mock bursting. The Ca_v3.2(+25) and exon 25 lacking (Ca_v3.2(–25)) splice variants display unique

Key points

- Burst-firing in reticular thalamic neurons is enhanced in the GAERS absence epilepsy model
- The frequency range of enhanced burst-firing correlates with SWDs
- A developmental increase occurs in the mutation-sensitive splice variant of Ca_v3.2
- Genetic knock-down of Ca_v3.2 shortens absence seizure duration but not seizure number in GAERS

biophysical properties and are expressed in approximately equal abundance in the thalamus of NEC rats.^{16,17}

Here we investigate the role of the TRN in absence seizure generation in GAERS by assessing the firing properties and T-type currents in intact TRN neurons. To achieve this, we elucidate the neurophysiological impact of the R1584P mutation in TRN neurons of GAERS and correlate it with developmental expression of the Ca_v3.2 (+25) splice variant. Furthermore, we show that selective knock-down of Ca_v3.2 channel expression in the GAERS TRN in vivo reduces the duration of seizures. This study demonstrates that Ca_v3.2 channels in the GAERS TRN play a key role in sustaining burst-firing in TRN neurons and that this is required for propagation of absence seizures.

2 | MATERIALS AND METHODS

All experiments were undertaken according to Canadian Council for Animal Care Guidelines. See Data S1 for detailed Materials and Methods.

3 | RESULTS

3.1 | Individual bursts in either epileptic or preepileptic GAERS do not display hyperexcitability

GAERS begin to express emergent SWDs around postnatal day (P) 30, and the epileptic phenotype is fully developed in all animals by P120.¹⁸ The excitability of NEC (n = 28) and GAERS (n = 20) TRN neurons was first assessed by analyzing single bursts using whole-cell current clamp in slices from adult rats (P120–P150). Individual bursts were induced in TRN neurons at their intrinsic V_m by incremental increases in depolarizing DC current injection for 1 second until the threshold for burst-firing was reached (Figure S1A). There were no differences detected for any single burst parameter in either fully epileptic GAERS or

NECs (current required for threshold bursting, number of action potentials per burst, resting membrane potential, input resistance, burst inflection point or latency to first action potential; Figure S1B-G; NEC $n = 28$ cells, $n = 13$ animals; GAERS $n = 21$ cells, $n = 12$ animals). We next examined single burst properties in P15-P20 TRN neurons to establish whether a hyperexcitable neuronal phenotype was present in the TRN neurons of GAERS prior to the expression of seizures. Like for adult rats, we found no differences between NEC ($n = 111$ cells, $n = 52$ animals) and GAERS ($n = 70$ cells, $n = 36$ animals) concerning single bursts induced by DC depolarization of TRN neurons from their intrinsic Vm or passive membrane properties (Figure S1B-G).

3.2 | Fully epileptic GAERS TRN neurons display hyperexcitable burst-firing in the GAERS SWD frequency range

During absence seizures, thalamic neurons display a rhythmic firing pattern of multiple bursts, sustained for the duration of the seizure.^{6,19} As such, TRN neurons were assessed over a series of multiple bursts at various frequencies (5-10 Hz) for a period of 2 seconds. This frequency range corresponds to spike frequencies that are at and just above or below those observed during SWDs in GAERS (~6-9 Hz).¹ To induce multiple bursting, a sinusoidal wave of hyperpolarizing/depolarizing current was injected at incrementally increasing amplitudes from each neuron's intrinsic Vm until burst-firing (>3 action potentials, >100 Hz) was observed in the initial 3 bursts of the series. As the series progressed, the oscillatory bursts induced by this protocol decreased in the number of action potentials per burst, in both NEC and GAERS (Figure 1A), likely a result of attenuated T-type currents across the series as channels accumulate in the inactivated state.^{12,16} To examine the degree of burst-firing sustained during the sinusoidal stimulation, a percentage was calculated by dividing the number of action potentials on the last burst by the number of action potentials on the first burst. In TRN neurons from fully epileptic GAERS (P120-P150), the number of action potentials on the last burst displayed no significant difference compared to NEC neurons at 5 Hz or 10 Hz (Figure 1B). However, within the GAERS SWD frequency band of 6-9 Hz, the number of action potentials per burst on the last burst in GAERS TRN neurons was significantly increased in comparison to NEC (Figure 1B). Together, these data indicate that fully epileptic GAERS TRN neurons possess an inherent ability to sustain burst-firing over a range of frequencies corresponding to the cycle frequency of SWDs recorded by EEG, compared to those from age-matched nonepileptic animals.

3.3 | Preepileptic GAERS TRN neurons display hyperexcitable burst-firing in a narrow frequency range

Burst-firing over a series of multiple bursts was next examined in TRN neurons from preepileptic (P15-P20) GAERS versus NEC rats (Figure 2). As in adults, the number of action potentials per burst decreased across the series of bursts in both NEC and GAERS preepileptic TRN neurons at all frequencies (Figure 2A). Unexpectedly, given that these experiments were undertaken in preepileptic animals, there was a significant increase in the number of action potentials on the last burst at 8-9 Hz in GAERS (Figure 2B). However, at lower frequencies (5-7 Hz) and at the higher frequency of 10 Hz, no difference in the number of action potentials on the last burst was observed. This indicates that TRN neurons in preepileptic GAERS (Figure 2B) can generate hyperexcitable burst-firing, albeit only at a comparatively narrow frequency range.

3.4 | T-type currents in GAERS TRN neurons display gain-of-function properties

TRN neurons express both the Ca_v3.2 and Ca_v3.3 T-type calcium channel subtypes at approximately equal levels.^{20,21} Upon exogenous expression, the GAERS Ca_v3.2 R1584P mutation selectively increases the rate of recovery from inactivation of Ca_v3.2(+25) variant channels.¹⁶ We hypothesized that native GAERS TRN neurons would also display gain-of-function T-type current biophysical properties. Whole-cell voltage-clamp was undertaken in TRN neurons, although limited by space-clamp considerations in acute brain slices from older animals, T-type calcium currents were recorded from acute brain slices from neonatal (P7-P9) NEC and GAERS rats (in the presence of sodium, potassium and HVA calcium channel blockers; TTX [600 nM], 4-AP [5 mM], TEA [10 mM], Cs⁺ [120 mM], conotoxin MVIIC [3 μM], SNX-482 [100 nM], Cd²⁺ [10 μM], and nimodipine [5 μM]). GAERS TRN neurons displayed a modest but significant increase in T-type current density in the range of -55 to -40 mV (NEC [$n = 6$], GAERS [$n = 6$], $P = .03-.04$ *t* test; Figure S2A), with no apparent differences in the voltage dependences of activation or inactivation between the 2 strains (Figure S2B). The rate of recovery from inactivation was also significantly increased in GAERS TRN neurons, displaying greater fractional recovery from 160 to 640 msec interpulse intervals (Figure S2C) and a significant decrease in the fast time constant of inactivation when fitted individually with a decaying double exponential (NEC = 232.7 ± 14.6 [$n = 11$ cells, $n = 5$ animals], GAERS = 182.7 ± 12.9 msec [$n = 9$ cells, $n = 4$ animals]; $P = .01$ *t* test).

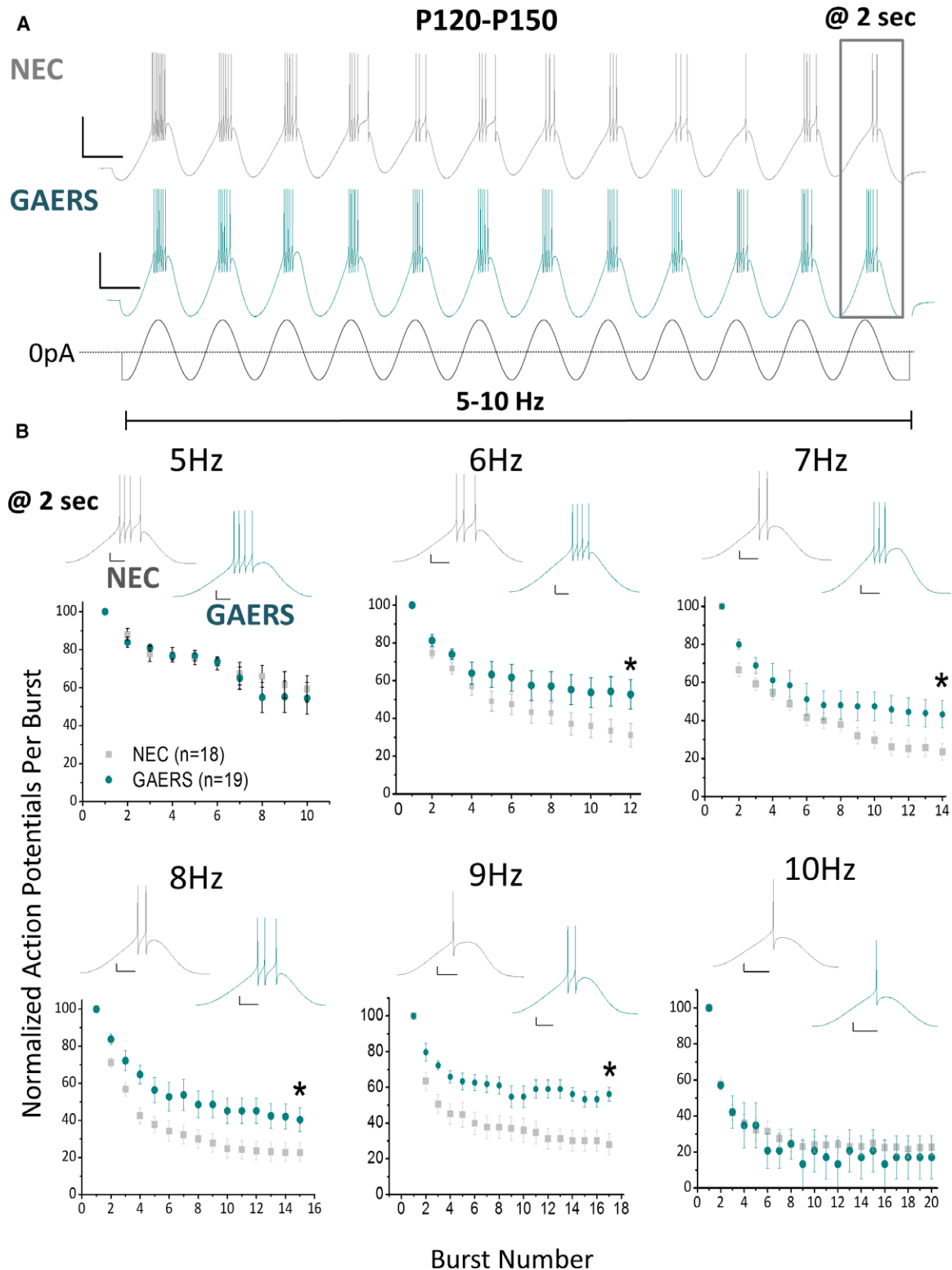


FIGURE 1 Adult GAERS TRN neurons display sustained oscillatory burst-firing at 6-9 Hz. A, Representative traces showing response of adult NEC (A—upper panel) and GAERS (A—middle panel) TRN neurons to a 2 second sinusoidal current injection stimulus (A—lower panel). Stimulus magnitude was incrementally increased until bursting (>3 action potentials) occurred on 3 initial bursts. Scale bars = 30 mV, 100 msec. B, The sinusoidal stimulus was applied at variable frequencies (5-10 Hz), with the number of action potentials per burst decreasing with time. Insets show representative voltage traces of the last burst in a series. 6 Hz: NEC = $31.2 \pm 6.2\%$, GAERS = 52.8 ± 7.8 , $P = .034$ *t* test; 7 Hz: NEC = $23.6 \pm 4.4\%$, GAERS = $43.3 \pm 7.2\%$, $P = .044$ *t* test; 8 Hz: NEC = $22.7 \pm 5.1\%$, GAERS = $40.3 \pm 6.4\%$, $P = .047$ *t* test; 9 Hz: NEC = $30.1 \pm 5.9\%$, GAERS = $56.2 \pm 3.7\%$, $P = .005$ *t* test. NEC (n = 20 cells, n = 11 animals), GAERS (n = 15 cells, n = 10 animals). Scale bars represent 20 msec and 10 mV. * $P < .05$

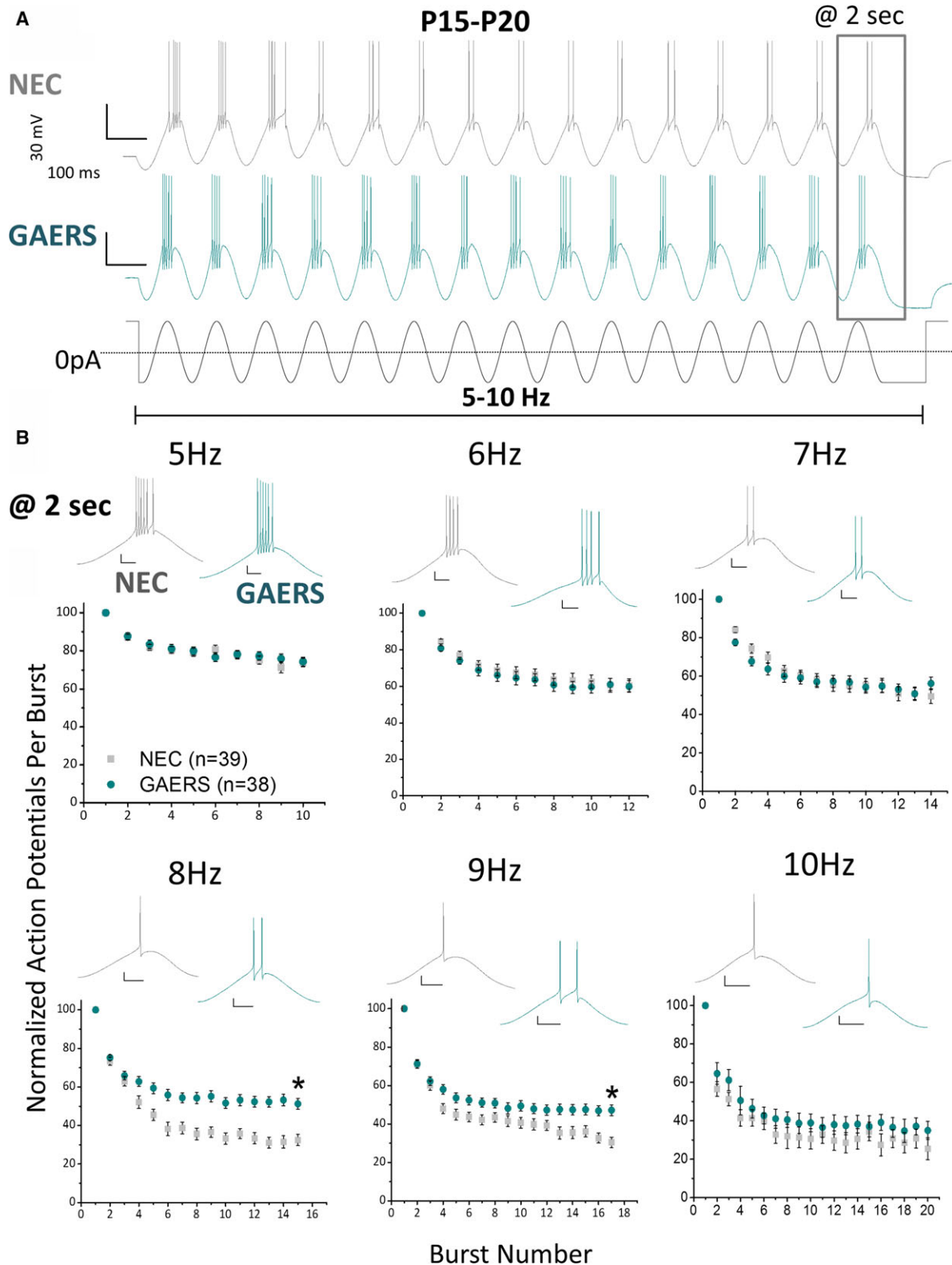


FIGURE 2 P15-P20 GAERS TRN neurons display sustained oscillatory burst-firing at 8-9 Hz. A, Representative traces showing response of P15-P20 NEC (A—upper panel) and GAERS (A—middle panel) TRN neurons to a 2-second sinusoidal current injection stimulus (A—lower panel). Stimulus magnitude was incrementally increased until bursting (>3 action potentials) occurred on 3 initial bursts. Scale bars = 30 mV, 100 msec. B, The sinusoidal stimulus was applied at variable frequencies (5-10 Hz), with the number of action potentials per burst decreasing with time. Insets show representative voltage traces of the last burst in a series. 8 Hz: NEC = $32.5 \pm 2.9\%$, GAERS = $51.2 \pm 2.8\%$, $P = .00001$ *t* test; 9 Hz: NEC = $30.5 \pm 2.7\%$, GAERS = $47.2 \pm 2.8\%$, $P = .00006$ *t* test; NEC (n = 32 cells, n = 20 animals), GAERS (n = 32 cells, n = 16 animals). Scale bars = 20 msec and 10 mV. * $P < .05$

To further understand the frequency-dependent contribution of T-type conductance to burst-firing, isolated T-type calcium currents were recorded in voltage-clamp while burst-firing waveforms were applied that were previously recorded from an adult GAERS TRN neuron in current clamp at 5 and 8 Hz (Figure S2D). The charge transference (taken from the integral of the current vs time per burst) was normalized to the whole-cell capacitance to account for variability in neuron size and provide a value of charge transference density, similar to the method of normalizing whole-cell currents for variability in cell size. Charge transference density was significantly increased for the last burst at 2 seconds in GAERS compared to NEC TRN neurons at 8 Hz (Figure S2E), but not at 5 Hz (Figure S2F). This confirms the presence in GAERS of a frequency-dependent, T-type calcium current gain-of-function using voltage-clamp in a physiological milieu at an early developmental stage.

3.5 | $\text{Ca}_v3.2(+25)$ expression increases with development

The GAERS genome possesses a gain-of-function missense mutation in the $\text{Ca}_v3.2$ gene.¹⁶ To assess whether this mutation affects the thalamic expression of calcium channel $\alpha 1$ subunits between GAERS and NEC during postnatal development quantitative real-time polymerase chain reaction (qRT-PCR) analyses were undertaken in samples of whole thalamus excised from preepileptic P10 and P120 epileptic rats. In these stages, thalamic expression of 8 of the 10 calcium channel $\alpha 1$ subunits was detected, including all 3 T-types ($\text{Ca}_v3.1$, $\text{Ca}_v3.2$, $\text{Ca}_v3.3$). Of note, within each subunit type there were no significant differences in overall relative expression between NEC and GAERS at the developmental stages examined (Figure 3A). Previously, we showed that the GAERS R1584P mutation produced a selective gain-of-function phenotype in $\text{Ca}_v3.2(+25)$ splice variant channels with a concomitant modest loss-of-function in $\text{Ca}_v3.2(-25)$ variant channels.¹⁶ The developmental expression of the $\text{Ca}_v3.2(+25)$ and $\text{Ca}_v3.2(-25)$ splice variants were assessed in samples of whole thalamus from P10, P20, and P120 animals using qRT-PCR and corrected to display splice-variant transcript copy number (see Data S1). Figure 3B shows that within each stage, the NEC and GAERS strains express equal amounts of $\text{Ca}_v3.2(+25)$ transcripts. This was accompanied by a significant developmental increase in the overall copy number expression of $\text{Ca}_v3.2(+25)$ transcripts in both NEC and GAERS between preepileptic (P10 and P20) thalamic samples compared to fully epileptic (P120) thalamus (Figure 3B). Contrastingly, thalamic expression of the $\text{Ca}_v3.2(-25)$ variant was significantly lower in GAERS compared to NEC at all 3 stages examined. In addition, NEC animals displayed a developmental decrease in $\text{Ca}_v3.2(-25)$ expression at P20 and P120

compared to P10, whereas in GAERS, a decrease was observed at P20 compared to P10 and P120. Together, the differences in relative splice-variant expression gives rise to a consistently greater ratio of $\text{Ca}_v3.2(+25)/\text{Ca}_v3.2(-25)$ transcripts in GAERS versus NEC animals through the course of development (Figure 3C). Furthermore, the $\text{Ca}_v3.2(+25)/\text{Ca}_v3.2(-25)$ transcript expression ratio is significantly different between all developmental stages in both NEC and GAERS (for clarity, significance between developmental stages for each strain is not shown in Figure 3C). The developmental increase in the mutation-sensitive $\text{Ca}_v3.2(+25)$ splice variant, together with hyperexcitable, sustained burst-firing in older GAERS provides a mechanism for the emergence of seizures in this model.

3.6 | Pharmacological blockade of the $\text{Ca}_v3.2$ T-type calcium channel abolishes sustained burst-firing in GAERS TRN neurons

To confirm whether the GAERS $\text{Ca}_v3.2$ channel was responsible for the increased TRN burst-firing properties, we removed the contribution of $\text{Ca}_v3.2$ -mediated calcium conductance by pharmacological blockade. Oscillatory current clamp experiments using 8-Hz sinusoidal stimuli were performed in slices from P15-P20 NEC and GAERS in the presence of nickel (Ni^{2+} ; 100 μM) to block $\text{Ca}_v3.2$, while allowing the majority of the $\text{Ca}_v3.3$ channels to remain unblocked and contribute to bursting (Figure S3).²² In the presence of Ni^{2+} , no significant effect was seen on passive membrane properties, such as resting V_m or input resistance (not shown); however, burst-firing required significantly greater current injection to achieve threshold in both NEC and GAERS TRN neurons (Figure 4A-C) as the contribution of $\text{Ca}_v3.2$ was removed. Oscillatory burst-firing at 8 Hz was then assessed by comparing the number of action potentials on the last burst of a 2-second oscillatory stimulation, relative to the first burst. It is notable that in the presence of Ni^{2+} there was no significant difference in sustained burst firing in GAERS TRN neurons compared to NEC, thereby abolishing the hyperexcitable phenotype (Figure 4A,D).

$\text{Ca}_v2.3$ (R-type) calcium channels are also sensitive to Ni^{2+} ; therefore, we sought to confirm the result with a second $\text{Ca}_v3.2$ channel inhibitor. Ascorbate (Asc; 300 μM) inhibits $\text{Ca}_v3.2$ channels in TRN neurons with little effect on $\text{Ca}_v3.3$, thought to occur via oxidation of a redox sensitive amino acid specific to $\text{Ca}_v3.2$.^{21,23} Asc was efficacious at attenuating burst threshold in most, but not all, TRN neurons ($n = 7$ of 10 NEC and $n = 6$ of 7 GAERS). The “non-responding” neurons were not included in further analysis, and we speculate that $\text{Ca}_v3.2$ channels in these neurons existed in a fully oxidized state prior to Asc application. In the remaining TRN neurons, Asc increased the current

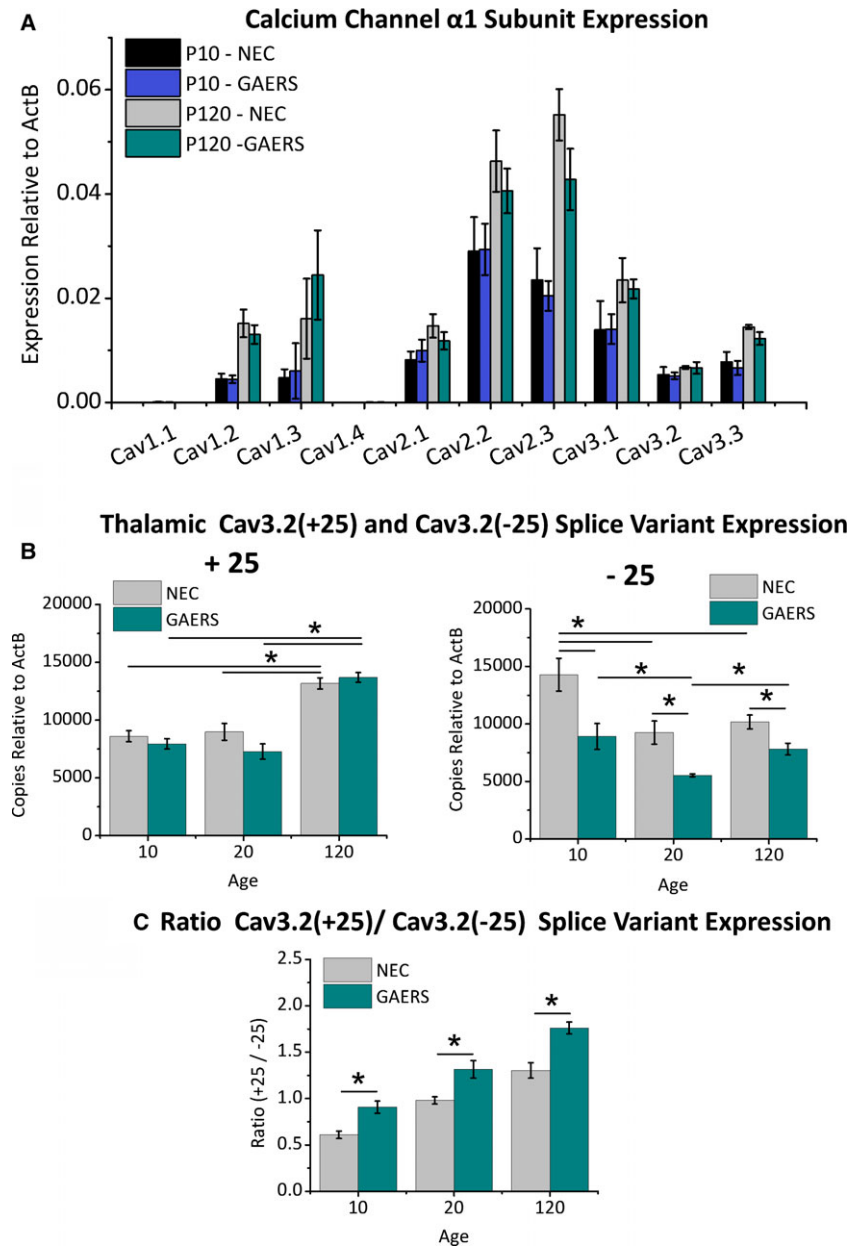


FIGURE 3 GAERS show a developmental increase in the expression of the Cav3.2(+25) splice variant. A, The relative mRNA expression of the T-type $\text{Ca}_v3.1$ - $\text{Ca}_v3.3$ and the high-voltage activated $\text{Ca}_v1.1$ - $\text{Ca}_v1.4$ and $\text{Ca}_v2.1$ - $\text{Ca}_v2.3$ Ca^{2+} channels was determined using qRT-PCR, comparing isoform expression between P10 and P120 in thalamic tissue from GAERS and NEC animals. Amounts are relative to Actin-B as a control. B, Using splice variant-specific probes for $\text{Ca}_v3.2(+25)$ and $\text{Ca}_v3.2(-25)$, the relative number of transcript copies of $\text{Ca}_v3.2(+25)$ and $\text{Ca}_v3.2(-25)$ splice variants was determined across development in GAERS and NEC animals ($n = 3$ animals per strain): $\text{Ca}_v3.2(+25)$: P10 NEC = 8601.2 ± 475.7 , P10 GAERS = 7944.6 ± 443.8 ; P20 NEC = 8985.0 ± 726.4 , P20 GAERS = 7278.1 ± 654.4 ; P120 NEC = 13177.8 ± 487.5 , P120 GAERS = 13692.3 ± 424.0 ; [P10 NEC vs P120 NEC] $P = .003$, [P20 NEC vs P120 NEC] $P = .005$ ANOVA; [P10 GAERS vs P120 GAERS] $P = .0006$, [P20 GAERS vs P120 GAERS] $P = .0003$ ANOVA. $\text{Ca}_v3.2(-25)$: P10 NEC = 14279.6 ± 1421.0 , P10 GAERS = 8910.9 ± 1125.8 , $P = .04$ t test; P20 NEC = 9246.1 ± 1006.7 , P20 GAERS = 5524.7 ± 214.0 , $P = .02$ t test; P120 NEC = 10170.6 ± 599.2 , P120 GAERS = 7814.7 ± 513.8 , $P = .04$ t test; [P10 NEC vs P20 NEC] $P = .035$, [P10 NEC vs P120 NEC] $P = .045$ ANOVA; [P10 GAERS vs P20 GAERS] $P = .036$, [P20 GAERS vs P120 GAERS] $P = .04$ ANOVA. C, The ratio of $\text{Ca}_v3.2(+25)$ to $\text{Ca}_v3.2(-25)$ was determined from copy number analysis for each of the developmental time points from both GAERS and NEC animals. All experiments were performed using thalamus samples from 3 rats for each age group (P10, P20, or P120) and from each of GAERS or NEC animals: P10 NEC = 0.61 ± 0.04 , P10 GAERS = 0.91 ± 0.07 , $P = .02$ t test; P20 NEC = 0.98 ± 0.04 , P20 GAERS = 1.31 ± 0.09 , $P = .03$ t test; P120 NEC = 1.30 ± 0.08 , P120 GAERS = 1.76 ± 0.06 , $P = .01$ t test; [P10 NEC vs P20 NEC] $P = .009$, [P10 NEC vs P120 NEC] $P = .0004$, [P20 NEC vs P120 NEC] $P = .017$; [P10 GAERS vs P20 GAERS] $P = .02$, [P10 GAERS vs P120 GAERS] $P = .0005$, [P20 GAERS vs P120 GAERS] $P = .014$ ANOVA. * $P < .05$

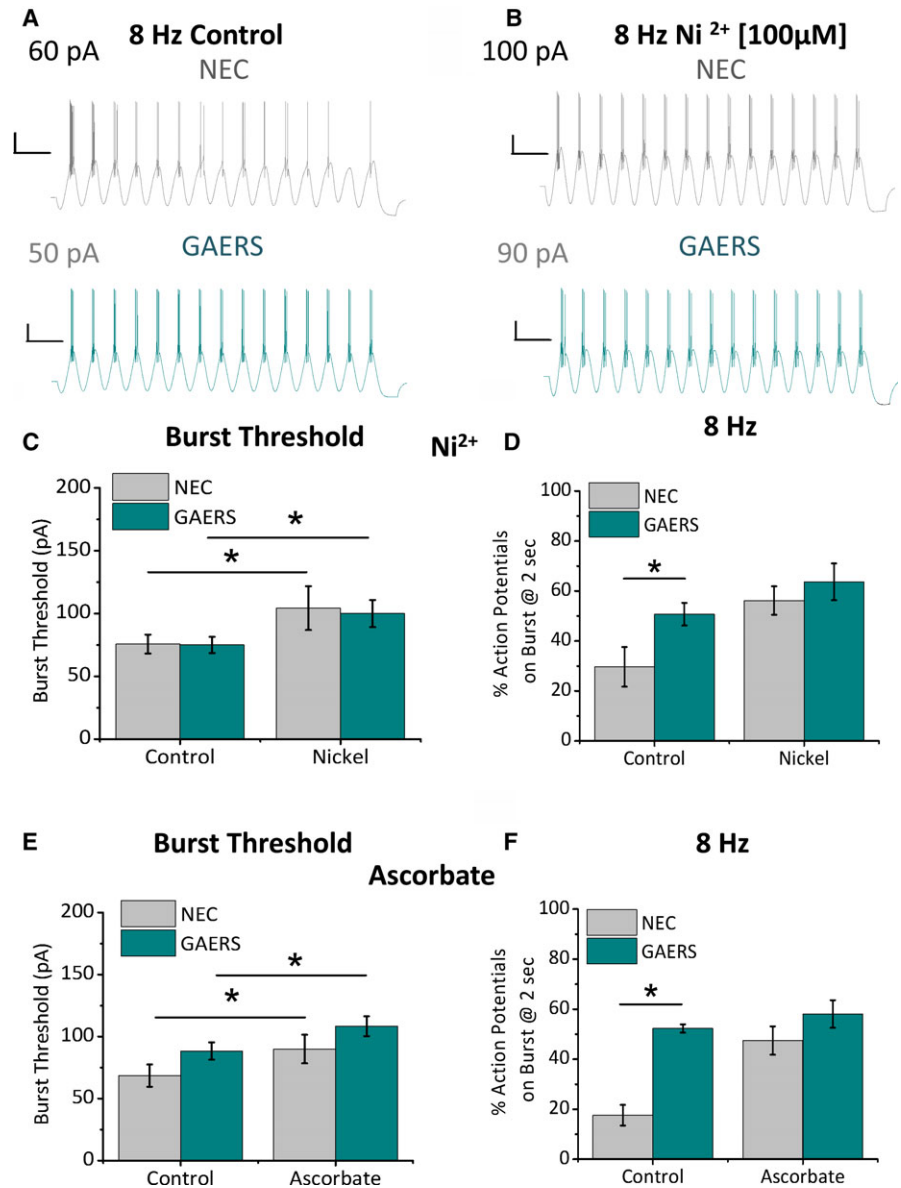


FIGURE 4 Sustained oscillatory burst-firing in P15-P20 GAERS TRN neurons is normalized by pharmacological $\text{Ca}_v3.2$ blockade. Representative traces show an oscillatory burst series in response to a 2-second sinusoidal current injection at 8 Hz in P15-P20 NEC (A, B—upper panels) and GAERS (A, B—lower panels) TRN neurons. Traces show burst-firing before (control; A) and after 10-minute application of Ni^{2+} (100 μM , B) via the perfusate to block $\text{Ca}_v3.2$. Histograms display mean data for burst threshold (C, E) and number of action potentials on the last burst of a 2-second stimulation, relative to the first burst (D, F) before (control) and after application of Ni^{2+} (100 μM , C, D; Control: NEC = $29.7 \pm 7.9\%$, GAERS = $50.7 \pm 4.5\%$ [$n = 8$], $P = .03$ t test, Ni^{2+} : NEC = $56.2 \pm 5.7\%$, GAERS = $63.7 \pm 7.3\%$, $P = .15$ t test, NEC [$n = 8$ cell, $n = 3$ animals], GAERS [$n = 8$ cells, $n = 3$ animals]) and ascorbate (300 μM , E, F; Control: NEC = $17.6 \pm 15.3\%$, GAERS = $52.3 \pm 1.7\%$, $P = .001$ t test, Asc: NEC = $47.5 \pm 5.7\%$, GAERS = $58.1 \pm 5.5\%$, $P = .22$ t test, NEC ($n = 8$ cells, $n = 3$ animals), GAERS ($n = 6$ cells, $n = 3$ animals) to block $\text{Ca}_v3.2$ in NEC and GAERS TRN neurons. * $P < .05$

injection threshold required to achieve burst-firing in both NEC and GAERS (Figure 4E). Furthermore, in the presence of Asc, GAERS TRN neurons did not show sustained oscillatory burst-firing at 8 Hz compared to NEC (Figure 4F).

3.7 | Selective thalamic knock-down of $\text{Ca}_v3.2$ in vivo effectively shortens seizures

The specific contributions of T-type calcium currents in TRN neurons toward absence seizures remain a subject of considerable investigation, and there have been no reports examining this issue in the GAERS model. As such, we next sought to assess the contribution of TRN $\text{Ca}_v3.2$ currents to seizure activity in vivo. The approach combined dicer small interfering (Dsi) RNAs targeted to $\text{Ca}_v3.2$ transcripts together with a recently developed methodology involving packaged lipid nanoparticles (LNPs) previously

demonstrated as an efficacious vector for in vivo knock-down.^{24,25} Initially, several distinct DsiRNAs were screened for their ability to attenuate exogenous $\text{Ca}_v3.2$ expression in transfected HEK cells (not shown) and subsequently on native $\text{Ca}_v3.2$ expression in cultured rat cortical neurons, with the best performing DsiRNA selected for packaging into LNPs containing DiI as a marker (Figure 5A,B). Control LNPs were generated by packaging DsiRNA for luciferase (Luc). $\text{Ca}_v3.2$ and Luc DsiRNA-containing LNPs were separately stereotactically injected bilaterally into the thalamus of P120-P150 GAERS (Figure 5C, see Experimental Procedures). Eight days to 9 days postinjection, TRN samples were isolated by dissecting from 3, 300- μm sequential, horizontal thalamic sections, and T-type calcium channel expression was analyzed by qRT-PCR. TRN slices from GAERS injected with $\text{Ca}_v3.2$ DsiRNA-LNPs displayed a selective knock-down of both $\text{Ca}_v3.2(+25)$ and $\text{Ca}_v3.2$

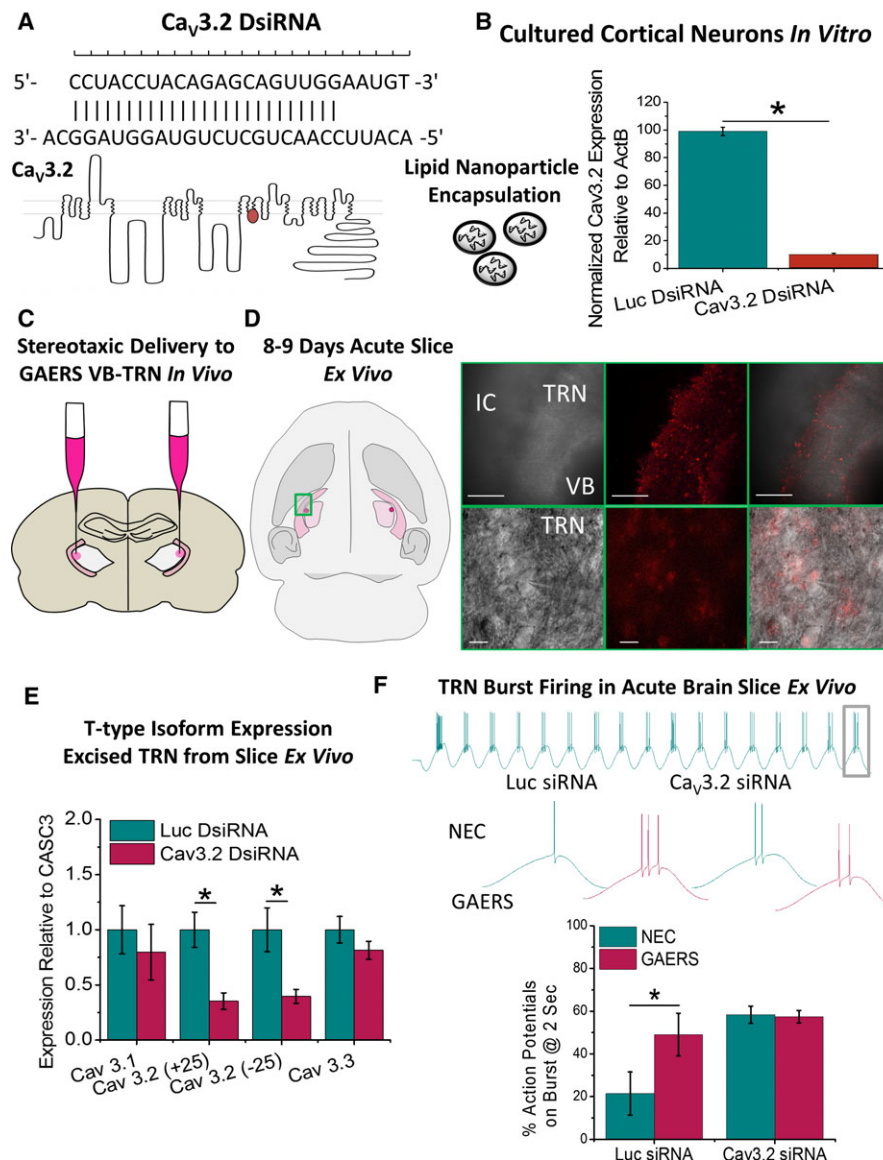


FIGURE 5 Selective knock-down of thalamic Ca_v3.2 in vivo. **A**, Dicer siRNA (DsiRNA) was designed to selectively anneal to the Ca_v3.2 channel and target for degradation. DsiRNA was packaged with DiI into LNPs for ApoE-mediated delivery to brain cells. **B**, Selective Ca_v3.2 knock-down was first confirmed in vitro by applying to cultured cortical neurons for 48 hours and expression levels assessed with qPCR. **C**, For in vivo analyses, P120-P150 GAERS rats were stereotaxically injected with luciferase control (Luc) or Ca_v3.2 DsiRNA LNPs bilaterally into the ventroposterior lateral thalamus to deliver DsiRNA to TRN neurons without damaging or lesioning the TRN. **D**, Following a 7- to 8-day recovery period, animals were euthanized, the brain removed, and LNP invasion of TRN was confirmed by DiI fluorescence ex vivo (*scale bars: top panels = 400 μm, bottom panels = 20 μm*). **E**, Ca_v3.1-Ca_v3.3 T-type isoform expression in TRN tissue was assayed using qPCR relative to the control gene CASC3. Histograms display mean data for expression of T-type calcium channel isoforms in TRN tissue dissected from 3 sequential 300-μm-thick horizontal brain slices for GAERS injected with Luc ($n = 6$ animals) and Ca_v3.2 DsiRNA ($n = 8$ animals); Ca_v3.1 isoform: Luc DsiRNA = 1.00 ± 0.22 , Ca_v3.2 DsiRNA = 0.79 ± 0.25 , $P = .55$ *t* test; Ca_v3.2(+25) splice variant: Luc DsiRNA = 1.00 ± 0.16 , Ca_v3.2 DsiRNA = 0.35 ± 0.07 , $P = .0017$ *t* test; Ca_v3.2(-25) splice variant: Luc DsiRNA = 1.00 ± 0.19 , Ca_v3.2 DsiRNA = 0.39 ± 0.06 , $P = .0067$ *t* test; Ca_v3.3 isoform: Luc DsiRNA = 1.00 ± 0.12 , Ca_v3.2 DsiRNA = 0.81 ± 0.08 , $P = .21$ *t* test. **F**, Oscillatory burst-firing was assessed in TRN neurons from acute thalamic brain slices in P19-P20 NEC and GAERS, 6-8 d following injection with either Luc ($n = 3$ animals, $n = 6$ cells) or Ca_v3.2 ($n = 3$ animals, $n = 7$ cells) siRNA. Bar charts show percentage number of action potentials on the last burst relative to the first burst from a 2-s oscillating wave stimulation at 8 Hz. * $P < .05$

(-25) splice variants, without significantly affecting the expression of the Ca_v3.1 or Ca_v3.3 T-type isoforms (Figure 5E). Expression of the control gene CASC3 relative to

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was not significantly affected in TRN tissue from Luc versus Ca_v3.2 DsiRNA-injected GAERS (Luc DsiRNA =

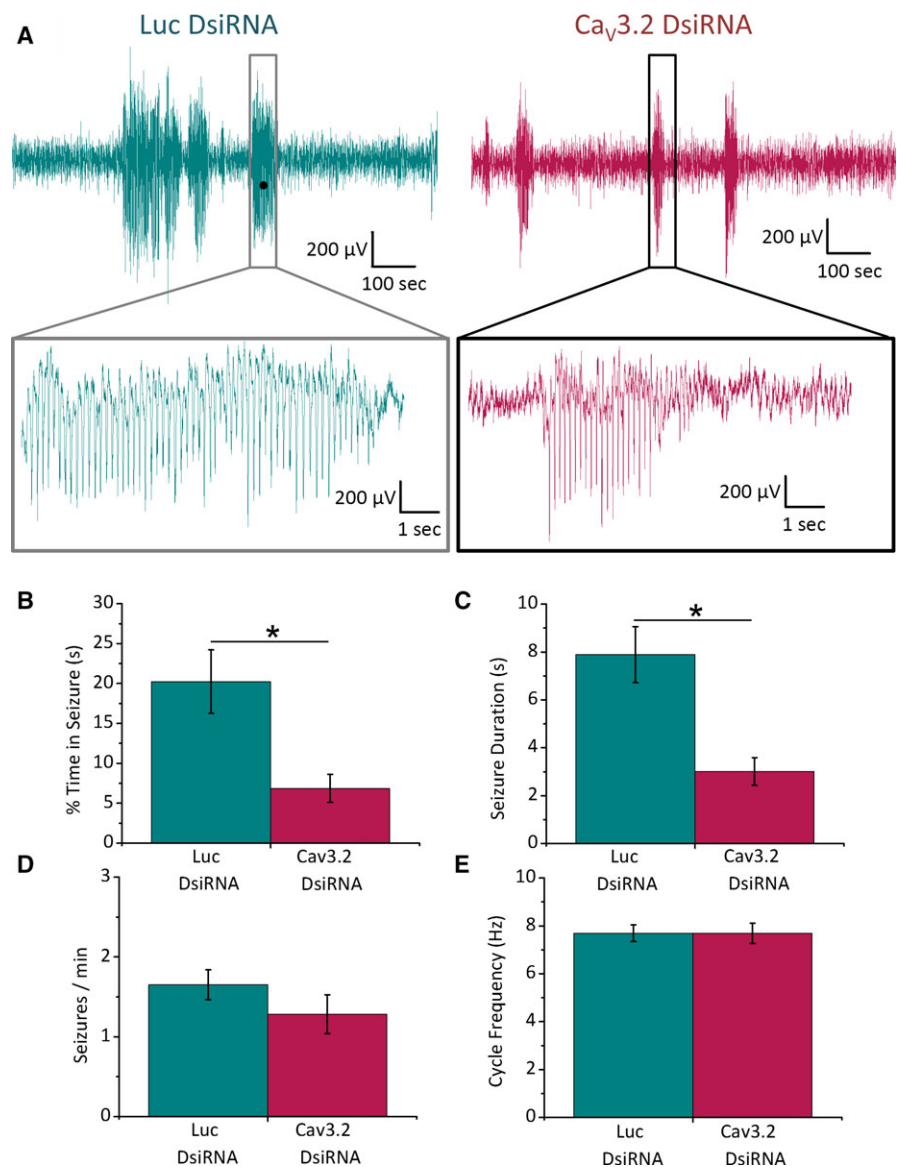
0.0083 ± 0.0005 , $\text{Ca}_v3.2$ DsiRNA = 0.0086 ± 0.0002 , $P = .56$ *t* test).

In a separate set of experiments, P11-P12 NEC and GAERS pups were stereotaxically injected with LNPs containing $\text{Ca}_v3.2$ or Luc DsiRNA into the thalamus (Figure S4). Following an 8- to 9-day period, acute brain slices were prepared, and neuronal transfection verified by the presence of DiI in cell bodies (Figure 5D). Oscillatory burst-firing at 8 Hz was again assessed by comparing the number of action potentials on the last burst of a 2-second oscillatory stimulation relative to the first burst. This confirmed that TRN neurons from GAERS injected with Luc displayed enhanced oscillatory burst-firing compared to NEC (as observed in Figures 3 and 6), seen as a significantly greater number of action potentials on the last burst (Figure 5F). Conversely, the hyperexcitable phenotype was not observed GAERS TRN neurons compared to NEC from animals injected with $\text{Ca}_v3.2$ DsiRNA. We did not

perform voltage-clamp experiments examining the effect of DsiRNA on T-type currents due to space-clamp limitations in TRN neurons from P15-P20 brain slices.

Seizure activity was also evaluated in GAERS P120-P150 animals injected with either Luc or $\text{Ca}_v3.2$ DsiRNA into the thalamus (Figure 6). Following a 7- to 8-day recovery period (the day before tissue extraction for mRNA analysis) free-moving electrocorticography (ECoG) recordings were performed using a wireless transmitter connected to a custom interface relaying signals from electrodes surgically implanted on the somatosensory cortex. Spontaneous SWDs were observed throughout the 60-minute recording period in both treatment groups (Figure 6A). However, compared to Luc DsiRNA-injected animals ($n = 6$), GAERS injected with $\text{Ca}_v3.2$ DsiRNA ($n = 8$) exhibited a significantly lower percentage time spent in the seizure state (Figure 6B) as well as significantly shorter seizure duration (Figure 6C). Conversely, the number of seizures

FIGURE 6 Thalamic $\text{Ca}_v3.2$ knock-down reduces seizure duration in GAERS. Adult GAERS rats were stereotaxically injected with $\text{Ca}_v3.2$ or Luc DsiRNA LNPs bilaterally into the VB thalamus, transfecting the TRN and implanted with skull screw electrodes on the somatosensory cortex connected to a custom EEG interface. A, Representative ECoG recordings from GAERS injected with Luc (left panel) or $\text{Ca}_v3.2$ (right panel) DsiRNA LNPs. Lower panels show ECoG data with expanded time resolution. Histograms show mean data with (B) % of total time spent in seizure state (Luc = $20.2 \pm 4.0\%$, $\text{Ca}_v3.2$ = $6.9 \pm 1.8\%$, $P = .005$), (C) seizure duration (Luc = 7.9 ± 1.1 s, $\text{Ca}_v3.2$ = 3.0 ± 0.6 s, $P = .002$), (D) number of seizures per minute (Luc = 1.65 ± 0.19 , $\text{Ca}_v3.2$ = 1.28 ± 0.24 , $P = .27$), and (E) SWD spike cycle frequency (Luc = 7.7 ± 0.3 Hz, $\text{Ca}_v3.2$ = 7.7 ± 0.4 Hz, $P = .99$) for GAERS rats injected with Luc ($n = 6$ animals) and $\text{Ca}_v3.2$ DsiRNA ($n = 8$ animals). * $P < .05$



occurring during the recording period was not significantly altered by $Ca_v3.2$ DsiRNA compared to Luc DsiRNA injection (Figure 6D). Furthermore, $Ca_v3.2$ and Luc DsiRNA-injected animals displayed no significant difference in SWD cycle frequency (Figure 6E).

4 | DISCUSSION

Although both increased T-type currents and an upregulation in $Ca_v3.2$ mRNA have been observed in GAERS TRN neurons,^{14,15} to our knowledge altered TRN burst-firing has not been thus far reported. GAERS TRN neurons have been shown to burst fire in synchrony with SWDs,^{6,19} and lesioning the TRN completely abolishes seizures,²⁶ confirming this region as an integral nucleus in the absence seizure-generating thalamocortical system. Here, we demonstrate that GAERS TRN neurons display a hyperexcitable oscillatory firing pattern, predicted to result from the gain-of-function induced by the $Ca_v3.2$ R1584P missense mutation.¹⁶ The hyperexcitability is progressively exacerbated during development and parallels an increased expression of the $Ca_v3.2(+25)$ splice variant sensitive to the R1584P mutation. We conclude that the developmental appearance of TRN hyperexcitability contributes to the age-dependent emergence of seizures in GAERS, due to a combined effect of the mutation's selective effect on $Ca_v3.2(+25)$ splice variant properties and the developmental increase in the relative abundance of this variant.

4.1 | GAERS TRN neurons display enhanced oscillatory burst-firing

To establish whether a gain-of-function in GAERS TRN T-type currents could induce a pathophysiologically relevant alteration to excitability we investigated TRN burst-firing properties. Although single burst characteristics were virtually identical between NEC and GAERS, we found enhanced oscillatory multiple burst-firing properties in GAERS TRN neurons. During multiple bursting in GAERS, the number of action potentials per burst attenuated to a significantly lesser degree compared to NEC animals. Furthermore, this occurred in a frequency and age-dependent manner with sustained burst-firing observed at a wide range of frequencies (6-9 Hz) in GAERS adults and corresponding to the frequency range of SWDs during GAERS seizures.²⁷ Contrastingly, preepileptic GAERS exhibited sustained burst-firing over a narrower frequency range (8-9 Hz). Together, these results demonstrate a developmental broadening of the input stimulation frequencies required to induce pathophysiological burst-firing that correlate with the emergence of absence seizures.

4.2 | $Ca_v3.2$ knock-down in GAERS TRN selectively affects seizure duration

We observed $Ca_v3.2$ -dependent enhanced oscillatory burst-firing in GAERS TRN neurons and predicted that the selective knock-down of $Ca_v3.2$ would alter absence seizure activity. Using intrathalamic injection of LNPs containing $Ca_v3.2$ DsiRNA, the expression of $Ca_v3.2$ transcripts in the TRN region was found to be reduced to approximately 35%-40% compared to that for Luc control-injected animals. Selectively decreasing $Ca_v3.2$ expression in the TRN significantly shortened seizure duration without significantly altering the number of seizures in GAERS. It is possible that a more pronounced degree of $Ca_v3.2$ knock-down in the TRN would result in a further decrease in seizure duration to the point where seizures cannot be sustained. Because no effect was observed following $Ca_v3.2$ knockdown on the number of seizures, the current results support the notion that absence seizures are initiated outside of the thalamus but are propagated by the TRN. In support, the initiating locus for absence seizures in both GAERS and WAG/Rij rats appears to be a cortical region involving the primary somatosensory cortex (S1Cx)²⁸⁻³¹ and S2/insular cortex.⁹ Our results support the theory, in that epileptic GAERS TRN neurons are shown to exhibit hyperexcitability (enhanced burst-firing) and therefore can propagate the oscillatory burst discharges that underlie absence seizures. Consistent with this notion, *in vivo* single-cell recordings have shown that physiological theta frequency oscillations (5-9 Hz) originating in the S1Cx of NEC rats briefly engage TRN neurons in oscillatory burst-firing, but that this is nonsustained, lasting <1 second.³² In contrast, GAERS cortical oscillations trigger sustained oscillatory burst-firing in TRN neurons, which is accompanied by SWDs on cortical EEG recordings and behavioral arrest in the animals.

Also to consider, the S1Cx region expresses all 3 T-type calcium channels, albeit only the layer V neurons intrinsically involved in thalamocortical network oscillations exhibit relatively higher expression of the $Ca_v3.2$ subtype.³³ As such, the gain-of-function effects of the GAERS R1584P mutation on $Ca_v3.2$ may also be reflected in the ability of $Ca_v3.2$ channels in the cortex to initiate or propagate oscillations in GAERS cortical pyramidal and/or interneurons. Putative epileptogenic burst-firing neurons have been observed in the GAERS somatosensory cortex,³¹ and global blockade of all 3 T-type calcium channels attenuates both the duration and number of seizures in addition to reducing cycle frequency.³⁴ Together with our data this provides strong evidence that seizures are initiated in the cortex and that the TRN acts to propagate seizures, thereby prolonging seizure duration.

4.3 | Altered Ca_v3.2 activity as a trigger for epileptic seizures

Absence seizures occur spontaneously in genetic animal models wherein T-type calcium channel activity is enhanced in the thalamus.³⁵ The case for involvement of the Ca_v3.2 subtype in the pathogenesis of epileptic seizures is increasingly strong. Within subpopulations of genetic generalized and childhood absence epilepsy patients a number of gain-of-function mutations have been identified in the *CACNA1H* gene encoding Ca_v3.2.³⁶ In addition, increased burst-firing in CA1 hippocampal pyramidal neurons during the spontaneous seizure development phase of the pilocarpine model of temporal lobe epilepsy appears to be due primarily to increased dendritic Ca_v3.2 expression.³⁵ In the electrical kindling model of temporal lobe epilepsy, increased T-type currents are also observed in CA1 neurons,³⁷ and the development of seizures is prevented by administration of a high-affinity T-type calcium channel blocker during the kindling phase.³⁸

It should be stated that selective genetic deletion of Ca_v3.2 and Ca_v3.3 channels in mouse TRN neurons abolishes burst-firing and has little effect on, or even exacerbates, pharmacological absence seizures generated by systemic administration of γ -butyrolactone (GBL).³⁹ We note, however that the GBL model does not induce typical absence seizures in mice.⁴⁰ Rather than displaying SWDs, the hallmark of absence seizures, mice injected with GBL and the neuroactive drug into which it is converted (γ -hydroxybutyric acid; GHB) demonstrate slow oscillating waves, lacking spikes and that are more comparable to a hypnotic state. Furthermore, GHB is a known γ -aminobutyric acid (GABA) agonist and mimics the native contribution of the GABAergic TRN neurons by hyperpolarizing thalamocortical neurons, thus a functional TRN is intrinsically redundant in this pharmacological model. Accordingly, it must be considered that the effects we observe in GAERS as related to Ca_v3.2 and the TRN may be specific to the GAERS model. Indeed, we speculate that absence seizures may be generated by hyperexcitability in any locus in the thalamocortical system by a number of means that enhance burst-firing. The mechanistic spread observed in different animal models most likely reflects a variety of polygenic, underlying causes in patients.

In summary, the current study demonstrates that the splice variant-sensitive and larger/faster recovering Ca_v3.2 currents induced by the R1584P mutation in GAERS are linked to abnormally sustained oscillatory burst-firing properties in TRN neurons. This allows TRN neurons to more readily engage in the pathological thalamocortical network oscillations that underlie absence seizures through corticoreticular hyperexcitability. Furthermore, the epileptic

phenotype in GAERS becomes apparent as the natural expression of the R1584P mutation-sensitive Ca_v3.2(+25) splice variant increases developmentally, thereby increasing in severity as animals age. In addition, we validate LNP-mediated delivery of DsiRNA as an effective methodology to selectively manipulate pathophysiological network activity in the brain. Finally, the study highlights the need for the development of selective T-type calcium channel blockers for the treatment of seizures in epilepsy.

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AUTHOR CONTRIBUTIONS

S.M.C. designed experiments, performed in vivo and in vitro electrophysiology recordings, sampled brain tissue for mRNA analysis, performed animal surgeries, designed seizure analysis software, and co-wrote the manuscript. J.R.T. performed mRNA and qPCR analyses and designed DsiRNA constructs. L.Y. performed animal surgeries. J.M.L. designed seizure analysis software. P.C.L. and P.R.C. provided the DsiRNA packaged into LNPs, and H.B.C., R.K., L.P.B. and R.L.R. assisted with in vitro testing of LNPs. K.L.P. and T.O.B. provided NEC and GAERS animals and edited the manuscript. B.A.M. assisted with editing of the manuscript. T.P.S. designed experiments, analyzed data, and co-wrote the manuscript. All authors participated in editing of the manuscript.

DISCLOSURE

The authors declare no competing financial interests. 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

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