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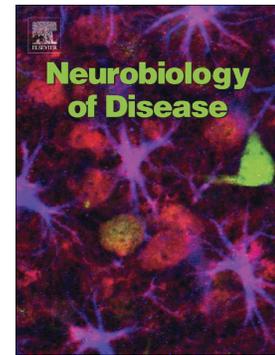
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**Effects Of The T-Type Calcium Channel $Ca_v3.2$ R1584P
Mutation On Absence Seizure Susceptibility In GAERS And NEC
Congenic Rats Models**

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Abstract

Rationale: Low-voltage-activated or T-type Ca^{2+} channels play a key role in the generation of seizures in absence epilepsy. We have described a homozygous, gain of function substitution mutation (R1584P) in the $\text{Ca}_v3.2$ T-type Ca^{2+} channel gene (*Cacna1h*) in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS). The non-epileptic control (NEC) rats, derived from the same original Wistar strains as GAERS but selectively in-bred not to express seizures, are null for the R1584P mutation. To study the effects of this mutation in rats who otherwise have a GAERS or NEC genetic background, we bred congenic GAERS-*Cacna1h*NEC (GAERS null for R1584P mutation) and congenic NEC-*Cacna1h*GAERS (NEC homozygous for R1584P mutation) and evaluated the seizure and behavioural phenotype of these strains in comparison to the original GAERS and NEC strains.

Methods: To evaluate seizure expression in the congenic strains, EEG electrodes were implanted in NEC, GAERS, GAERS^{-*Cacna1h*NEC} without the R1584P mutation, and NEC^{-*Cacna1h*GAERS} with the R1584P mutation rats. In the first study, continuous EEG recordings were acquired from week 4 (when seizures begin to develop in GAERS) to week 14 of age (when GAERS display hundreds of seizures per day). In the second study, the seizure and behavioural phenotype of GAERS and NEC^{-*Cacna1h*GAERS} strains were evaluated during young age (6 weeks of age) and adulthood (16 weeks of age) of GAERS, NEC, GAERS^{-*Cacna1h*NEC} and NEC^{-*Cacna1h*GAERS}. The Open field test (OFT) and sucrose preference test (SPT) were performed to evaluate anxiety-like and depressive-like behaviour, respectively. This was followed by EEG recordings at 18 weeks of age to quantify the seizures, and spike-wave discharge (SWD) cycle frequency. At the end of the study, the whole thalamus was collected

for T-type calcium channel mRNA expression analysis.

Results: GAERS had a significantly shorter latency to first seizures and an increased number of seizures per day compared to GAERS^{-Cacna1hNEC}. On the other hand, the presence of the R1584P mutation in the NEC^{-Cacna1hGAERS} was not enough to generate spontaneous seizures in their seizure-resistant background. 6 and 16-week-old GAERS and GAERS^{-Cacna1hNEC} rats showed anxiety-like behaviour in the OFT, in contrast to NEC and NEC^{-Cacna1hGAERS}. Results from the SPT showed that the GAERS developed depressive-like in the SPT compared to GAERS^{-Cacna1hNEC}, NEC, and NEC^{-Cacna1hGAERS}. Analysis of the EEG at 18 weeks of age showed that the GAERS had an increased number of seizures per day, increased total seizure duration and a higher cycle frequency of SWD relative to GAERS^{-Cacna1hNEC}. However, the average seizure duration was not significantly different between strains. Quantitative real-time PCR showed that the T-type Ca²⁺ channel isoform Cav3.2 channel expression was significantly increased in GAERS compared to NEC, GAERS^{-Cacna1hNEC} and NEC^{-Cacna1hGAERS}. The presence of the R1584P mutation increased the total ratio of Cav3.2 +25/-25 splice variants in GAERS and NEC^{-Cacna1hGAERS} compared to NEC and GAERS^{-Cacna1hNEC}.

Discussion: The data from this study demonstrate that the R1584P mutation in isolation on a seizure-resistant NEC genetic background was insufficient to generate absence seizures, and that a GAERS genetic background can cause seizures even without the mutation. However, the study provides evidence that the R1584P mutation acts as a modulator of seizures development and expression, and depressive-like behavior in the SPT, but not the anxiety phenotype of the GAERS model of absence epilepsy.

Introduction

Childhood absence epilepsy is the most common form of genetic generalized epilepsy (GGE)¹. Absence seizures, the pathognomonic seizure type, appear during early childhood and are characterized by spike-wave discharges (SWDs) in the EEG². Absence seizures also occur in other generalized epilepsy syndromes, including juvenile absence epilepsy and juvenile myoclonic epilepsy.

T-type Ca^{2+} channels in the thalamocortical circuitry play a key role in the development of the SWDs in humans and animal models³⁻⁹. T-type Ca^{2+} channels generate Ca^{2+} spikes near resting membrane potential, which can result in burst firing and oscillatory behavior in the thalamocortical circuitry^{10-12, 13}. This role of T-type Ca^{2+} channels on the thalamocortical circuitry has been supported by the increased mRNA expression of the $\text{Ca}_v3.2$ ¹⁴ and T-type Ca^{2+} currents¹⁵ in the reticular nucleus of the thalamus (nRT) of the Genetic Absence Epilepsy Rats from Strasbourg (GAERS). In addition, increases in T-type currents have been observed preceding the development of absence seizures in SNAP-25 KO mice¹⁶. To further support the importance of the T-type calcium channels in absence seizures, we have previously shown that Z944, a selective high-affinity pan-T-type Ca^{2+} channel antagonist, significantly inhibits absence seizures by 85-90% in the GAERS model¹⁷.

Moreover, mutations in the gene that codes for the human $\text{Ca}_v3.2$ channel (*CACNA1H*) have been described in patients with GGE and absence seizures^{7, 18-25}. Exogenous expression of mutant human $\text{Ca}_v3.2$ channels in cultured cells reveals various biophysical changes to channel functioning, such as prolonged time course of the activation/inactivation of the calcium channels²⁶⁻³⁰. A previous study from our group identified a splice variant dependent $\text{Ca}_v3.2$ (R1584P) gain of function mutation in GAERS that was not present in non-epileptic control (NEC) rats that had been bred from the same original Wistar colony⁵. Alternative

splicing of exon 25 creates two splice variants (SV) of Cav3.2: (+25) which contains exon 25, and (-25), which lacks that exon. When exon 25 is present, the channel recovers faster from inactivation and has greater Ca²⁺ charge transference during burst firing conditions. Significantly, the R1584P mutation segregates with seizures in the F₂ progeny rats produced from double-crossing GAERS and NEC, as described in ⁵. Animals with two copies of the R1584P mutation spend more time having seizures, as evaluated on the EEG, than animals null for the mutation ⁵.

Affective disorders such as depression and anxiety are common comorbidities present in people with absence epilepsy, and they occur at frequencies substantially greater than those observed in appropriately matched people without epilepsy ³¹⁻³⁴. These commodities have also been described in the GAERS model³⁵. Some lines of evidence show that modulation of T-type calcium channel function can have anti-depressant ³⁶⁻³⁸ and anxiolytic effects^{39 40}.

To further evaluate the role that the Cav3.2 *Cacn1h* (R1584P) mutation plays in the epilepsy and behavioral phenotype seen in GAERS we created congenic rat strains. A congenic strain involves the transference of a specific gene or phenotypic trait to a new genetic background by repetitive back-crossing ⁴¹. In standard or classical congenic breeding, each generation is selected using marker assisted genotyping for those animals that inherit the segment of interest and have the greatest homozygosity for the recipient strain alleles in all other regions ^{42, 43}. If one assumes a 50% contribution of genetic material from each parent at each back-cross, at least 10–12 generations of backcross breeding will be needed to generate an inbred strain that has 99.9% homology with the parental strain ^{42, 44}.

Congenic strains eliminate much of the genetic background interference that could confound the effect of the mutation of interest on the phenotype under investigation. Such strains are bred so that they are essentially isogenic with the parent strain, except for the region derived

from the other parental strain ⁴⁵. Here, we exploit congeneric models to explore the specific role played by the R1584P mutation in: A) the expression of seizures from young animals to adulthood; and B) in the anxiety-like and depressive-like phenotype of the animals when the mutation is present in the NEC non-epileptic genetic background, or absent in GAERS with epilepsy prone genetic background.

Journal Pre-proof

Materials and methods

Breeding of the congenic strains

The congenic strains were bred at the University of Rennes, France. GAERS, homozygous (+/+) for R1584 mutation, were crossed with NEC, null (-/-) for the mutation, to generate F₁. Subsequently, F₁ are interbred to create a F₂ generation. The F₂ offspring were then genotyped for the mutation and bred back again to the GAERS or NEC (N₁ GAERS x F₂ or N₁ NEC x F₂, respectively). After 12 generations of backcrossing (N₁₂), both lines of animals (N₁₂ GAERS x F₂) and (N₁₂ NEC x F₂) would be heterozygous (+/-) for the *Cacna1h* R1584P mutation. Then, an intercross between heterozygous (+/-) x (+/-) from each line is performed. We then proceeded to select the *Cacna1h* R1584P (-/-) from the N₁₂ GAERS x F₂ line and named them GAERS-Cacna1h^{NEC} (*i.e.* a GAERS strain without the R1584P mutation), and rejected the R1584P -/+ and +/+ subjects. Conversely, from the N₁₂ NEC x F₂ line, we selected the *Cacna1h* R1584P +/+ and named them the NEC-Cacna1h^{GAERS} (*i.e.* a NEC strain with a homozygous R1584P mutation). Congenic strains are maintained with a brother x sister mating procedure. Ear biopsies from the breeders and pups (at the time of weaning), were collected for genotype analyses as detailed below.

Animals and ethical statement

Male GAERS, NEC, GAERS-Cacna1h^{NEC} and NEC-Cacna1h^{GAERS} were used in all the experiments. Experimental procedures were approved by The Florey Animal Ethics committee (ethics numbers 1011823 and 14-053-UM) and adhered to the Australian Code for the care and use of animals for scientific purposes. All the experiments were conducted by researchers blinded to the experimental conditions. Animals were individually housed with alternating 12-hour cycles of light and dark. Food and water were provided *ad libitum* for the whole duration of the study. The GAERS and NEC lines used for sequencing are at F₂₅ and

F₁₈ generations, respectively. The congenic used for this project were from GAERS-Cacna1h^{NEC} and NEC-Cacna1h^{GAERS} N₁₂F₇ filial generations.

Sample size analysis

Power analyses based on our previously published GAERS studies^{13,5}, indicated that an n=6 per group was required to detect a 30% difference between the groups considering $\alpha=0.05$ and power=95%.

Experimental timeline

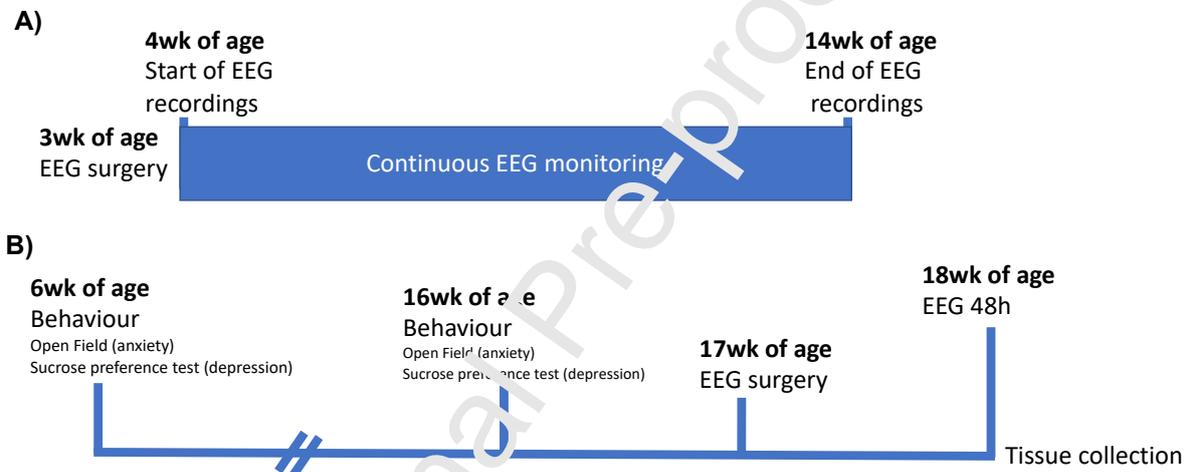


Figure 1. Experimental timeline. Two experiments were performed. The first in **A)** *Seizure expression across the lifespan of congenic strains* evaluated the expression of seizures from young age (4 weeks) to adulthood (14 weeks of age). The second one is **B)** *Seizure and behavioral phenotype of GAERS and NEC-Cacna1hGAERS strains during young age and adulthood.*

Identification of the genotypes of the GAERS, NEC and congenic strains.

Ear biopsies were collected at weaning and sent for genotyping for the R1584P variant Transnetyx (Cordova, TN, USA) using the forward (5'- GAA CCA CAA CCC CTG GAT GC -3') and reverse (5'- CCT GCG CCT CCT CTC CAG -3') primers⁵.

Seizure expression across the lifespan of the congenic rats

EEG electrode implantation surgery

Three-week-old NEC (n=6), GAERS (n=6), GAERS-Cacna1h^{NEC} (n=6) and NEC-Cacna1hG^{AERS} (n=6) were anesthetized with isoflurane (Ceva isoflurane, Piramal Enterprises Limited, India), and EEG Surgery was performed under aseptic technique as described previously^{46, 47}. EEG recording electrodes (EM12/20/SPC, Plastics One Inc) were inserted via burr holes without penetrating the dura, one on each side of the frontoparietal region (AP: ± 1.7 ; ML: -2.5) and two to each side of the temporal area (AP: ± 5.6 ; ML: left 2.5). Ground and reference electrodes were implanted on each side of the parietal bone above the cerebellum^{48, 49} and were secured using dental cement (VX-SC1000GVD5/VX-SC1000GMLLQ, Vertex, Australia) around the electrodes and over the skull. Buprenorphine (0.05 mg/kg SC, Indivior Australia) was used as an analgesic, at the start of the surgery, and every 12 hours for 3 days after the surgical procedure⁴⁸.

Continuous EEG monitoring

One week after the EEG surgery, animals were connected using cables (M12C-363, Plastics One Inc, Australia)³⁶. Video EEG (vEEG) recordings were acquired continuously using Profusion 3 software over 11 weeks (from 4 weeks to 14 weeks of age) (Compumedics, Australia) unfiltered and digitized at 512Hz⁵⁰. The age of the start of recordings was selected based on previous studies showing that the development of the seizures in GAERS increases with the age of animals. Seizures start to occur from around 4 weeks of age until early adulthood at about 14 weeks of age, which might be due to the maturation of thalamocortical circuitry^{51, 52}.

Seizure analyses

All vEEG recordings were analyzed using Assyst, a semi-automated seizure detection

software (Assyst, Australia)⁴⁶. An absence seizure was defined as spike and wave discharge (SWD) of more than three times baseline amplitude, a frequency of 6-12 Hz, and duration of >2 seconds on the EEG recording^{53, 46}. No SWD-like events were found with a frequency < 6Hz. The start and end of each seizure were determined by manually marking the beginning and end of each SWD on Assyst, determined by the first/last spike between the seizure and the background EEG. Two blinded independent experts visually confirmed seizure events. The total number of seizures, total seizure duration, and average seizure duration were quantified for all animals.

Behavioral and seizure phenotype of GAERS and NEC-Cacna1h^{GAERS} strains

Behavioral tests

NEC (n=11), GAERS (n=12), GAERS-Cacna1h^{NEC} (n=12) and NEC-Cacna1h^{GAERS} (n=12) underwent behavioral tests to evaluate anxiety and depressive-like behavior at 6 weeks of age (young age) and 16 weeks of age (adulthood) as described in Figure 1. All behavioral tests were performed in a light-controlled (~110 lux), closed, quiet and clean room between 10 am and 4 pm. The animals had at least 1h to acclimatize to the room before the start of any of the behavioral tests. All behavioral tests were performed with the investigator blinded to the congenic strains and completed at least 24h apart. All the behavioral tests were recorded and analyzed using EthoVision (version 3.0.15, Noldus)^{35, 36}.

Open field test

The open field test (OFT) is a 100cm circular arena, with an inner circle area of 66 cm diameter virtually defined using EthoVision. The rat is placed gently into the center of the field, and its behavioral activity is monitored for 10 min. The distance travelled as well as the entries and time spent in the inner circle were recorded^{35, 36}.

Sucrose preference tests

In the sucrose preference test (SPT), animals were presented with two bottles, one filled with water and the other filled with 2% sucrose in water and allowed to drink freely for 24 hours^{50, 54, 55}. On both tests, bottle position was randomized to avoid position preference^{35, 36, 56}. The volume and weight of the solution in the bottle were measured before and after each trial's completion; total fluid intake and percentage preference for sucrose were recorded.

EEG surgery, acquisition, and analysis

EEG surgery was performed at 17 weeks as described for the continuous EEG monitoring experiments. After 7 days of recovery, EEG was acquired for 24 hours and analyzed as described above.

Analysis of spike cycle frequency within a discharge

EEG recordings were analyzed for the cycle frequency of the SWDs using Clampex 10.2 software (Molecular Devices, Sunnyvale, CA, U.S.A.). For each trace, the mean cycle frequency within a discharge was calculated from 10 seizures with a minimum of 100 spikes per seizure and a minimum latency interval of 5 minutes between each seizure start time. If traces with less than 10 seizures matched the criteria, then seizures with shorter latency intervals or seizures with fewer events were analyzed⁵⁷.

Tissue collection

Animals were anesthetized using 5% isoflurane (Ceva isoflurane, Piramal Enterprises Limited, India), then culled using a lethal dose of i.p. Lethabarb (150mg/kg; pentobarbitone sodium; Virbac, Aus.) 48 hours after EEG recordings. Whole thalamus was rapidly dissected and snap-frozen in liquid nitrogen. The samples were stored at -80°C for further molecular analysis.

Quantitative PCR of T-type calcium mRNA expression

RNA was extracted using the RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's protocol. RNA concentration and purity were determined using spectrophotometric readings with the NanoDrop 3000 spectrophotometer (NanoDrop Technologies). 1µg RNA was reverse transcribed to cDNA using the Omniscript RT Kit (QIAGEN), following the manufacturer's protocol. The synthesized cDNA was stored at -20 °C. T-type calcium channel expression and $Ca_v3.2$ +25 and -25 splice variant expressions were assessed using quantitative polymerase chain reaction (qPCR). Each 10µl reaction volume consisted of 25ng of cDNA, 5µl of TaqMan Universal PCR Master Mix, and catalogued TaqMan gene expression assays for $Ca_v3.1$ (Assay ID Rn00581051_m1, Applied Biosystems), total $Ca_v3.2$ (Assay ID Rn01460348_m1, Applied Biosystems), and $Ca_v3.3$ (Assay ID Rn01505208_m1, Applied Biosystems). For the gene expression analysis of the $Ca_v3.2$ splice variants, custom-designed Taqman gene expression assays were used: + exon 25 (H- $Ca_v3.2$ -plus25 forward primer, CCGCAGGAGCACTTTCC; H- $Ca_v3.2$ -plus25 reverse primer, AGTGTGTGAATAGTCTGCGTAGTA; H- $Ca_v3.2$ -plus25-Probe, CCAACCCAGAGGCCAG), - exon 25 (H- $Ca_v3.2$ -minus25 forward primer, CGCCGGGAGGAGAAACC; H- $Ca_v3.2$ -minus25 reverse primer, AGTGTGTGAATAGTCTGCGTAGTA; H- $Ca_v3.2$ -minus25-Probe, CTGGGCCTTCCTGCGCC) ^{5, 36}. Relative expression of the $Ca_v3.1$, total $Ca_v3.2$, $Ca_v3.3$, $Ca_v3.2$ splice variant +25 and -25 SV were compared to the geometric average of the mRNA levels of the housekeeping genes: *Rpl1* (Rn03467157_gH), *Rpl13a* (Rn00821946_g1) and *Gapdh* (Rn01775763_g1). Analysis was performed using the $\Delta\Delta$ CT method ⁵⁸. The average of the relative expression levels was compared between the NEC, GAERS, GAERS- $Cacna1h^{NEC}$ and NEC- $Cacna1h^{GAERS}$.

Statistical analysis

Normal distribution was assessed using the Shapiro–Wilk test. For normally distributed data, unpaired T-test (total seizure duration, average seizure duration, spike frequency) or one-way ANOVA with Bonferroni *post-hoc* (6-week-old OFT, adult SPT, Cav3.1-3.3 mRNA expression) were used. Non-normally distributed data were analyzed using Mann-Whitney test (latency to the first spontaneous seizure, total number of seizures) or Kruskal-Wallis test with Dunn's *post-hoc* (6-week-old SPT, adult OFT and SV ratio mRNA expression). Repeated measures two-way ANOVA with Šídák's multiple comparisons *post-hoc* was used to analyze the seizure expression of the continuous EEG cohort using GraphPad Prism 9 (GraphPad Software, Inc. USA). Statistical significance in all cases was set at $p \leq 0.05$.

Results

Genotyping confirms the presence of the R1584 in the GAERS and NEC-Cacna1h^{GAERS} strain

Genotyping was carried out in all the animals involved in this study. Genotyping confirmed that all the GAERS and the NEC-Cacna1h^{GAERS} rats were homozygous for the R1584P mutation, whereas all the NEC and GAERS-Cacna1h^{NEC} rats were null for the mutation.

The presence of the R1584P mutation promotes the appearance of seizures at a younger age in GAERS but is not sufficient to generate absence seizures in NEC-Cacna1h^{GAERS}.

The latency to the first spontaneous seizure in the GAERS, homozygous for the R1584P mutation, was detected at a significantly younger age ($P = 0.002$, average 4.83 weeks, range 4-8 weeks) versus the GAERS-Cacna1h^{NEC} without the R1584P mutation (first seizure detected at an average 12.33 weeks, range 10-14 weeks, Figure 2A). Repeated measures two-way ANOVA revealed that GAERS displayed a significantly increased number of seizures per day in comparison to GAERS-Cacna1h^{NEC} [time x strain interaction $F(10, 100) = 4.055$, $P = 0.0001$; time effect $F(3.158, 31.58) = 10.99$, $P < 0.0001$; strain effect $F(10, 100) = 27.80$,

P<0.0001, Figure 2B). Post-hoc multiple comparison analysis revealed a significant difference between the number of seizures of GAERS and GAERS-Cacna1h^{NEC} during weeks 10-14. In contrast, the presence of the R1584P mutation in the NEC-Cacna1h^{GAERS} was not enough to produce spontaneous seizures in their seizure-resistant background. The morphology of the SWD GAERS-Cacna1h^{NEC} was very similar to that of the GAERS. Examples of GAERS and the GAERS-Cacna1h^{NEC} SWDs are presented in Figure 2C and 2D, respectively. As expected, NEC rats did not display any absence seizure for the study duration.

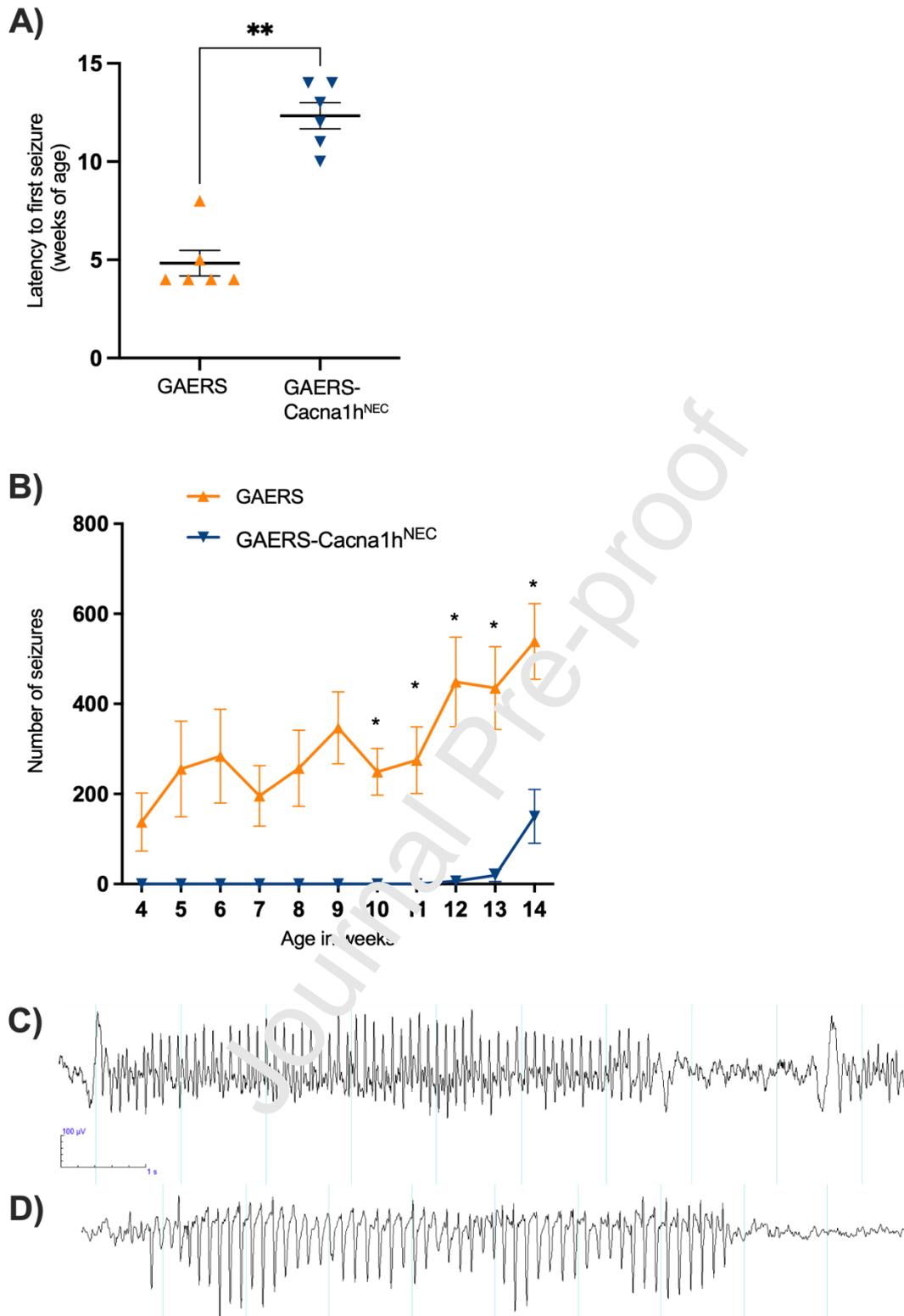


Figure 2. The effects of the R1584P mutation on seizure expression during the week 4 and week 14 postnatal in congenic strains. GAERS rats showed **A)** reduced latency to the first seizure (**, $P < 0.01$, Mann-Whitney test) and an increased **B)** average of seizures per day during different postnatal weeks (*, $P < 0.05$, indicates post hoc differences between average number of seizures, repeated measures two-way ANOVA with Bonferroni's post hoc) in comparison to GAERS-Cacna1h^{NEC} during the 11 weeks of continuous. NEC-Cacna1h^{GAERS} and NEC rats did not show any seizures during the study duration and are not included in both graphs. C) Example of a GAERS SWD and D) GAERS-Cacna1h^{NEC}. Data shown as mean \pm S.E.M.

The R1584P mutation modifies the frequency and characteristics of the SWD.

Consistent with our long-term EEG recordings findings, the NEC and NEC-Cacna1h^{GAERS} did not display any seizures at 18 weeks of age. Analysis of the EEG showed that the GAERS had an increased number of seizures per day ($P < 0.001$, Figure 3A) and total seizure duration ($P < 0.01$, Figure 3B) relative to GAERS-Cacna1h^{NEC}. The average seizure duration was not significantly different between strains (Figure 3C). However, GAERS showed a significantly higher cycle frequency of SWD than the GAERS-Cacna1h^{NEC} ($P < 0.0001$).

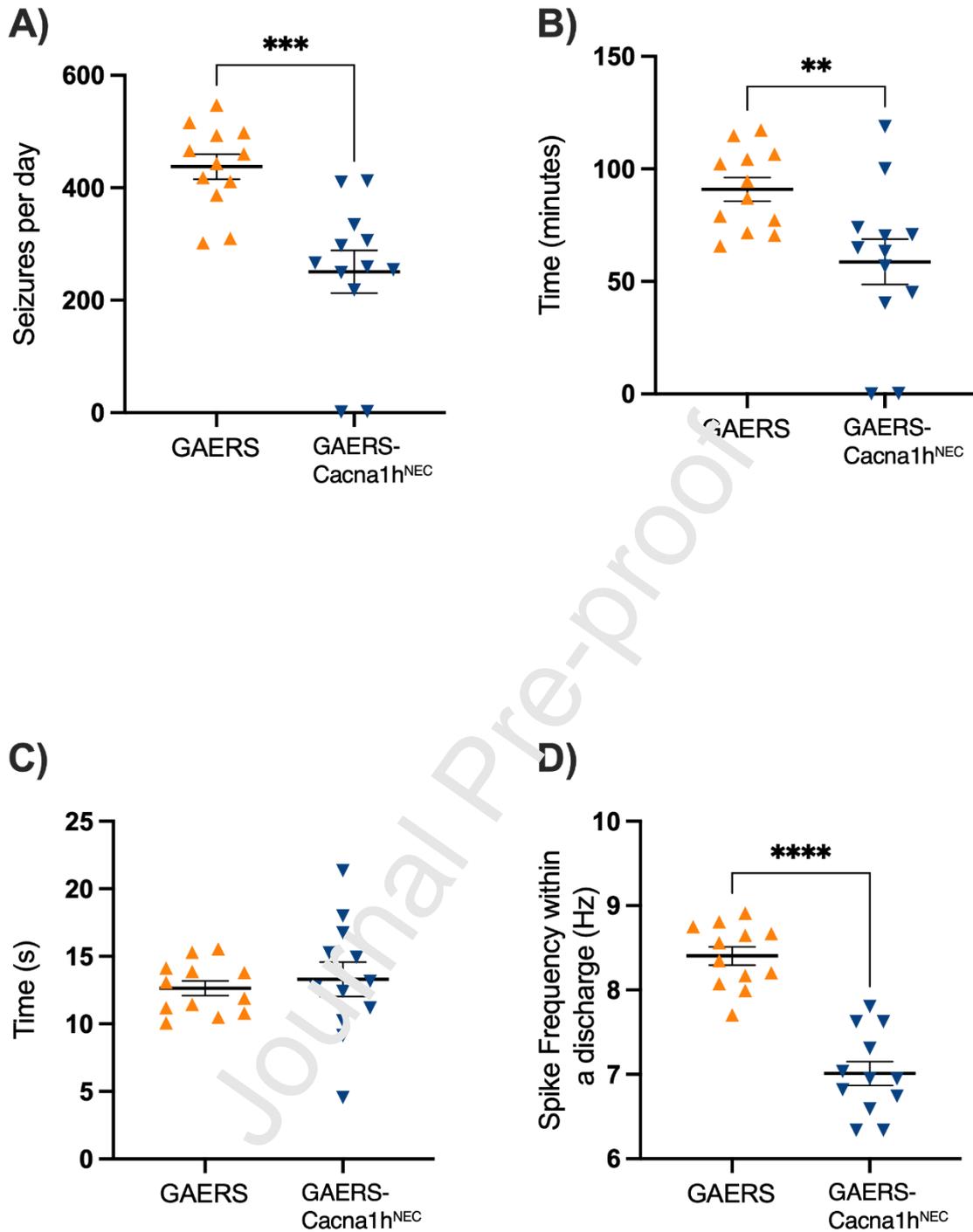


Figure 3. Effects of the R1584P mutation on the seizure expression and SWD characteristics. EEG recordings were analyzed for **A)** number of seizures per day, **B)** total seizure duration, **C)** average seizure duration and **D)** spike frequency per discharge. (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; Mann-Whitney test for A; Unpaired t-test for B-D). Data shown as mean \pm S.E.M.

Young and adult GAERS and GAERS-Cacna1h^{NEC} display an anxiety-like behavior phenotype

For the second experiment, anxiety and depressive-like behavior was evaluated in 6 week-old young rats and again in adulthood at 16-week of age. Six-week-old GAERS and GAERS-Cacna1h^{NEC} rats spent significantly less time in the inner circle of the OFT compared to NEC and NEC-Cacna1h^{GAERS} ($P < 0.0001$ in all comparisons; Figure 4A), which is indicative of increased anxiety-like behavior³⁵. Similarly, 16-week-old GAERS and GAERS-Cacna1h^{NEC} rats spent significantly less time in the inner circle compared to NEC ($P < 0.01$ and $P < 0.05$, respectively; Figure 4B) and NEC-Cacna1h^{GAERS} ($P < 0.001$ and $P < 0.01$, respectively; Figure 4B). Distance travelled was not significantly different between strains.

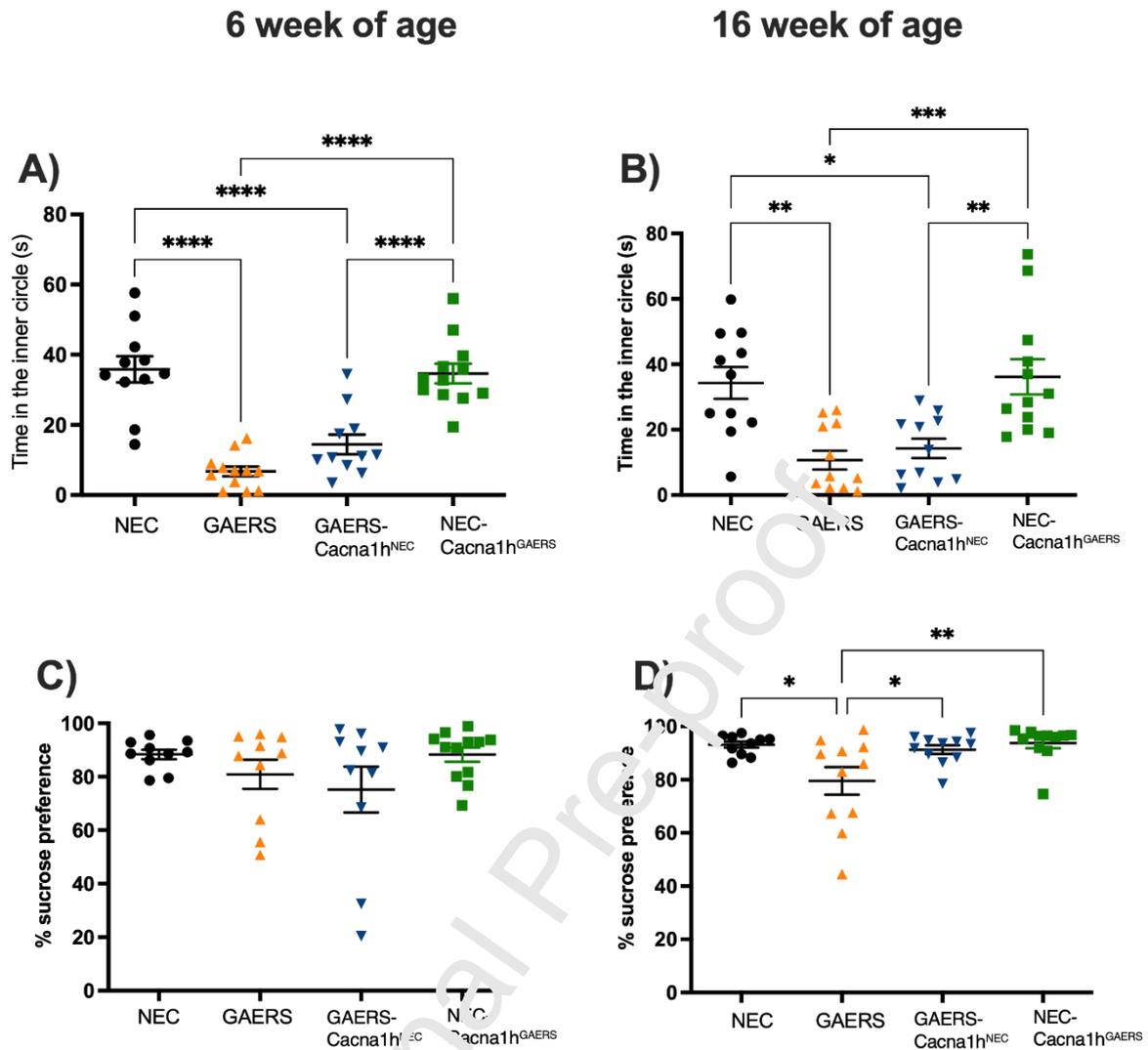


Figure 4. GAERS congenic display anxiety-like behavior but not depressive-like behavior. GAERS and GAERS-Cacna1h^{NEC} rats showed reduced time spent in the inner circle of **A) Open field test (OFT)** at 6 weeks and in the **B) OFT** at 16 weeks of age. **C) Sucrose preference test (SPT)** showed no difference between strains at 6 weeks of age. **D) SPT** at 16 weeks of age showed that only the GAERS, but not the GAERS-Cacna1h^{NEC}, displayed depressive-like behavior. (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; One-way ANOVA with Bonferroni's post hoc for A and D; Kruskal-Wallis test with Dunn's post-hoc for B and C). Data shown as mean \pm S.E.M.

The R1584P homozygous GAERS showed depressive-like behavior in adulthood.

The SPT is a measure of the ability of the animal to experience pleasure. An increased anhedonic state is one of the main clinical features of depression³⁵. Sucrose preference was calculated as the percentage of the total volume of the solutions drunk (both sucrose and water). Sucrose preference was not significantly different between strains at 6 weeks of age (Figure 3C). In contrast, results from the SPT showed that the GAERS develop a depressive-like behavior phenotype at 16 weeks of age and that the GAERS' sucrose preference was significantly reduced compared to NEC ($P < 0.05$, Figure 3D), GAERS-Cacna1h^{NEC} ($P < 0.05$) and NEC-Cacna1h^{GAERS} ($P < 0.01$).

T-type Ca²⁺ channel mRNA expression is altered in congenic strains.

Quantitative real-time PCR analysis of the thalamus from 17-week-old rats showed that the T-type Ca²⁺ channel isoform Cav3.1 (Figure 5A) and Cav3.3 (Figure 5C) were not significantly different between strains. However, Cav3.2 channel expression was significantly increased in GAERS compared to NEC ($P < 0.05$, Figure 5B), GAERS-Cacna1h^{NEC} ($P < 0.01$) and NEC-Cacna1h^{GAERS} ($P < 0.01$). The presence of the R1584P mutation increased the total ratio of Cav3.2 +25/-25 splice variants in GAERS and NEC-Cacna1h^{GAERS} compared to NEC ($P < 0.001$ for both comparisons; Figure 5D) and GAERS-Cacna1h^{NEC} ($P < 0.0001$ and $P < 0.001$, respectively).

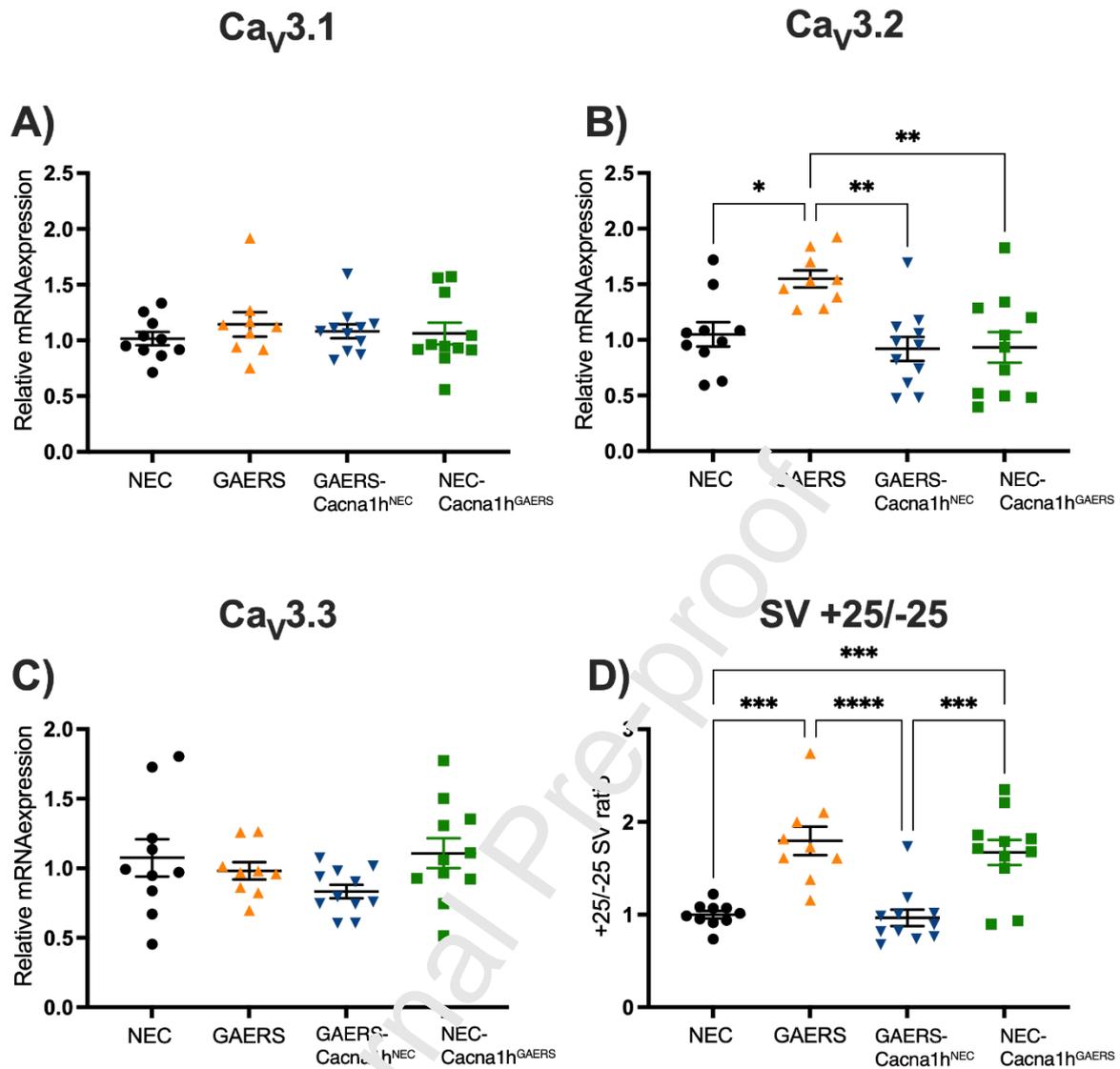


Figure 5. Relative mRNA expression of T-type calcium channels in the thalamus of adult rats. A) $Ca_v3.1$, B) $Ca_v3.2$ C) $Ca_v3.3$; D) $Ca_v3.2$ +25/-25 SV (*, $P < 0.05$; **, $P < 0.01$; *, $P < 0.001$; ****, $P < 0.0001$; One-way ANOVA with Bonferroni's *post-hoc* for A-C; Kruskal-Wallis test with Dunn's *post-hoc* for D). Data shown as mean \pm S.E.M.**

Discussion

Mutations in the gene encoding the T-type Ca^{2+} $Ca_v3.2$ isoform have been described in patients with absence epilepsy^{7, 18-24}, and GAERS^{5, 53}. Although the variants of human absence epilepsy are primarily missense, one frameshift⁵⁹ and one missense variant¹⁹ have been previously identified.

In this study, we utilized congenic rat strains to investigate the effects of the GAERS-specific *Cacna1h* R1584P gain of function mutation in isolation on different genetic backgrounds. In the NEC (null for the R1584P mutation) and the NEC-*Cacna1h*^{GAERS} strain (which carries two copies of the GAERS R1584P mutation on a NEC background) no seizures were detected⁵². In contrast, the GAERS (homozygous R1584P) and GAERS-*Cacna1h*^{NEC} strain (null for the R1584P mutation on a Ca_v3.2 GAERS background) expressed several seizures per day. Based on these data, it appears that the R1584P mutation might not be the only factor for the development of seizure by itself in GAERS, but could be a modulator of seizures development and expression. To evaluate this, we did a long-term follow-up, starting from week 4 of age, when GAERS are known to begin to develop absence seizures, to week 14, where all GAERS are epileptic displaying hundreds of SWDs^{51, 60, 61}. We found that GAERS-*Cacna1h*^{NEC} without the R1584P mutation develop seizures at a later age (early adulthood >10 weeks of age), and that they also displayed less seizures per day in contrast to GAERS.

It is well established that GGE is usually a polygenic condition both in humans and animal models^{5, 53, 62, 63}. This is supported by large scale whole genome and exome sequencing studies have reported multiple *de novo* and rare variants in people with epilepsy compared to non-epileptic controls^{64, 65}, including a recent study that failed to find variants of the Ca_v3.2 gene as the sole cause of monogenic human GGE¹⁹. Similarly, whole-genome sequencing revealed GAERS-specific variants, 25 annotated as stop codon gain/loss, 56 as putative essential splice sites, and 56 insertions/deletions that may contribute to the absence epilepsy phenotype⁵³. Moreover, a network medicine integrative study of multi-mics (proteomics and metabolomics) analysis identified over 240 differentially expressed proteins between GAERS and NEC, and enrichment in oxidative pathways, downregulation of the lysine degradation pathway and highlighted *Gstm1* and *Aldh2* as central regulatory hubs of these seizure-

associated pathways⁵⁴. Therefore other “seizure genes” from GAERS are necessary in combination to produce the epileptic phenotype. In addition, it is important to consider other important ion channels that may contribute to the absence seizure phenotype, for example alteration Hyperpolarization and Cyclic Nucleotide channel 1 (HCN1)^{66, 67}, sodium and potassium channels⁶⁸.

We have previously shown that the *Cacna1h* R1584P mutation segregated with the epilepsy phenotype of double-crossed NEC and GAERS⁵. However, some rats that were null for the mutation still displayed absence seizures, further supporting the polygenic mode of determinants of absence seizures in this model, and that the R1584P mutation is a seizure modulator instead of the sole contributor of seizure generation⁵. Moreover, up to 30% of the Wistar rats can present spontaneous SWDs, which become more prominent as the rat ages, while NEC do not have any absence seizures at all, even after more than 12 months of follow-up^{52, 61}. Therefore, it is plausible that the NEC and NEC-*Cacna1h*^{GAERS} genetic background contains genes that confer resistance to the development of absence seizures, which may suppress the pro-epileptic effect of the R1584P mutation. Moreover, it may be possible that the effects of the mutation on the epileptic phenotype of the GAERS are likely a result of epistatic gene-gene interactions^{63, 69} and, potentially, gene-environment interactions^{70, 71}.

As a result, any functional interactions between any GAERS-specific mutation(s) (*i.e.* R1584P variant) and their effect on seizure susceptibility and genetic background may be relevant to understanding the polygenic nature of idiopathic generalised epilepsy with absence seizures⁶³.

Together, our data demonstrate that the *Cacna1h* R1584P mutation in isolation on a seizure-resistant genetic background was insufficient to generate absence seizures, and that a GAERS

genetic background can cause seizures even without the mutation. The R1584P gain of function effects could potentially be a factor in the earlier development of absence seizures in GAERS in contrast to GAERS-Cacna1h^{NEC}, as low-threshold spikes generated by T-type Ca²⁺ channels have been shown to play an essential role in the generation of oscillatory thalamocortical rhythms and the switch between tonic and burst firing patterns in the thalamus⁷²⁻⁷⁵. Moreover, elevated T-type Ca²⁺ currents precede the onset of absence seizures in a SNAP-25-deficient mouse¹⁶. Temporal changes in Ca_v3.2 conductance can potentially synchronize epileptiform discharges¹⁰. Thus, the more prominent currents achieved by the R1584P mutation in Ca_v3.2 channels during high-frequency bursts may be sufficient to synchronize epileptiform discharges, which subsequently lower the threshold and facilitate the occurrence of early seizures. Moreover, the R1534I variant might render neurons of the thalamus more susceptible to excitatory corticothalamic inputs, producing more robust bursting activity, which could explain the early appearance and increased number of seizures in GAERS, homozygous for the mutation.

Interestingly, the presence of the R1584P mutation also modified the intrinsic SWDs characteristics, as GAERS had a higher spike cycle frequency within the SWDs than GAERS-Cacna1h^{NEC} without the mutation. This could be explained by the R1584P gain of function effects, which reduce the channel recovery time and generate a greater charge transference⁵, which would allow for a “faster” generation of SWD cycles. It is well known that the spike frequency within SWD is higher during the first few seconds of the seizure and then decreases to 7 Hz⁷⁶⁻⁷⁷. However, the average seizure duration was not significantly different between strains.

Pathogenic variants of the Ca_v3.2 coding gene *CACNA1H* have been reported in people with absence epilepsy, and exogenous expression of those mutant human Ca_v3.2 channels have

revealed a variety of biophysical changes^{29, 30, 28, 26, 27}. Other mutations in high voltage-activated non-T-type Ca^{2+} channels have been found to cause an absence epilepsy phenotype in monogenic rat models like *lethargic* and *tottering*⁷⁸⁻⁸⁰, as well as in humans with episodic ataxia type 2 and familial hemiplegic migraine type 1⁸¹⁻⁸³. However, monogenic disorders that have absence seizures as the primary seizure phenotype are associated with other disturbances, including ataxia and other cerebellar abnormalities in humans and rats. Importantly, in both ataxia type 2 and familial hemiplegic migraine type 1, seizures are not the main feature of the syndrome, and some patients do not develop seizures at all^{82, 84}. It may be possible that mutations in the $\text{Ca}_v3.2$ gene can contribute to an individual's susceptibility to epilepsy but are not sufficient to cause epilepsy by modulating the seizure threshold; however, $\text{Ca}_v3.2$ mutations might not be sufficient to cause seizure on their own^{20, 21, 23, 30, 59}. Nevertheless, no single mutation, including in the *CACNA1H* gene¹⁹, has been described as the sole cause of GGE with absence seizures, which agrees with our findings in GAERS.

The behavioral comorbidities in GAERS have been well characterized. However, it is unclear whether the phenotype is a consequence of the same genomic changes that determine the absence seizure phenotype, or due to other genetic variants that accompany the inbred selection of both strains⁸. Our results from the OFT were in concordance with our previous study where the anxiety phenotype of GAERS is manifested from an early age and persist to adulthood^{35, 86}. The GAERS-*Cacna1h*^{NEC} displayed a similar anxious phenotype to GAERS, but the variant on its own was unable to promote an anxious phenotype in the NEC-*Cacna1h*^{GAERS}. An interesting study performed by Marques-Carneiro et al in 2014⁸⁵ compared the behavioral phenotype of GAERS, NEC and their common parental strain, Wistar rats. They found that the NEC and GAERS are ends of the behavioral spectrum, GAERS displaying consistent depression and anxiety-like behavior, while NEC do not^{35, 85}.

At the same time, the GAERS anxiety phenotype was not significantly different from Wistars, but the NEC had a considerably less anxious phenotype than both Wistars and GAERS. While some studies have shown that abnormal T-type Ca^{2+} channel dependent-burst firing in the amygdala may regulate anxiety-like behavior³⁹ and that modulation of T-type Ca^{2+} channel function has anxiolytic effects⁴⁰; it may be possible that the *Cacna1h* R1584P mutation by itself was not enough to generate anxiety in a NEC anxiolytic genetic background

Genetic susceptibility to develop epilepsy and affective disorders have been well documented.^{87, 88} People with genetic epilepsy have an increased risk to develop depressive disorders^{89, 90}. The comorbid expression of these diseases raises the possibility that common or closely linked genes might be responsible^{90, 91}. We found that GAERS displayed the expected depressive phenotype only at 15 weeks of age. Interestingly, the absence of the R1584P mutation in GAERS-*Cacna1h*^{NEC} prevented them from showing the expected GAERS depressive phenotype³⁵. Still, the mutation on its own was not enough to elicit depressive-like behavior in NEC-*Cacna1h*^{GAERS}, indicating that the R1584P mutation was critical but not sufficient to manifest the depressive phenotype of GAERS. It may be possible that the depressive-like behavior in the GAERS-*Cacna1h*^{NEC} animals is a consequence of epilepsy, and it is aided by the presence of the T-type Ca^{2+} channel variant³⁹. The habenula is a phylogenetically conserved epithalamic brain structure that is known to regulate depressive-like behavior⁹², and part of its function is controlled by T-type Ca^{2+} dependent bursts activation^{37, 39, 93}. Emerging evidence has shown that pharmacological regulation of T-type Ca^{2+} function with Z944, a pan-T-type channel modulator, with higher affinity for $\text{Ca}_v3.2$, reduces depressive-like behavior in epileptic animals^{36, 37}. Moreover, ethosuximide, a non-specific T-type Ca^{2+} calcium channel antagonist, is in clinical trials for treatment-resistant depression³⁸.

CACNA1H has 12-14 alternative splice sites, and alternative splicing pathways produce both functional and non-functional $Ca_v3.2$ isoforms, which are likely to affect the membrane firing⁹⁴. Missense mutations have been shown to affect the activity of alternate spliced proteins⁹⁵. In GAERS, the functional effects of the R1584P mutation depend on alternative splicing of exon 25, only manifest in $Ca_v3.2 +25$ SV. Additionally, the ratio of $Ca_v3.2 +25/-25$ SV increased in GAERS compared to the NEC rats⁵. Similarly, the molecular analysis in the congenic strains revealed a significantly increased $Ca_v3.2 +25/-25$ SV ratio in the GAERS and NEC-*Cacna1h*^{GAERS} that have two copies of the R1584P mutation. These findings suggest a direct effect of the R1584P mutation on transcriptional regulation of the $Ca_v3.2 +25$ SV. Moreover, our study supports previous evidence that *Cav3.2* mRNA expression¹⁴ and T-type Ca^{2+} currents¹⁵ are elevated in the thalamus of GAERS. Literature has shown that intronic or exonic variants could lead to catastrophic splicing abnormalities, including exon skipping or activation of cryptic splice sites⁹⁶. As shown here, the R1584P mutation may alter the ratio of alternatively spliced $Ca_v3.2$ isoforms and have the potential to cause pathological changes and may facilitate the epileptic phenotype in GAERS.

On the other hand, this study is not without limitations. Firstly, even though our study evaluated the effects of the R1584P mutation up to 18 weeks of age, when all of the GAERS have hundreds of seizures per day, we cannot rule out that the NEC-*Cacna1h*^{GAERS} indeed won't develop SWD later in life. Still, it seems unlikely, considering the potential characteristics of the SWD-resistant NEC background. Secondly, depression and anxiety-like behavior are complex and can sometimes be difficult to evaluate in rodents. Even though we showed a robust phenotype in anxiety- and depressive-like phenotype in GAERS and anxiety-like behavior in GAERS-*Cacna1h*^{NEC}, complementary tests could have been used to further confirm the behavioral phenotype of the strains. For example, studies have questioned the reliability of the sucrose preference test to evaluate anhedonic-like behavior in

rodents^{97, 98} A progressive ratio schedule, where increasing effort is required to obtain a reward, could be used to corroborate our findings⁹⁸. Lastly, while GEARS gradually develop absence seizures, like humans, all of the GAERS continue to have absence seizures during adulthood, whereas the majority of the absence seizures are resolved in early adulthood.

The evidence provided in this study reinforces the notion that absence epilepsy is a complex polygenic disorder^{7, 62} and whilst T-type calcium channel Cav3.2 variants are not the sole cause of the absence of epilepsy in GAERS, as in humans¹⁰ they do have a critical role modulating the development and characteristics of epilepsy and depressive phenotype.

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Highlights

- GAERS had a significantly shorter latency to first seizures, increased number and duration of seizures, and higher cycle frequency of SWD compared to congenic GAERS without the R1584P T-type Ca^{2+} mutation mutation (i.e. GAERS-Cacna1h^{NEC}).
- The homozygous presence of the R1584P variant in congenic NEC rats (i.e. NEC-Cacna1h^{GAERS}) was not enough to generate spontaneous seizures.
- GAERS and GAERS-Cacna1h^{NEC} rats developed anxiety-like behaviour in contrast to NEC and NEC-Cacna1h^{GAERS} at 6 weeks and 16 weeks of age, indicating that the R1584P variant was not a determinant of this behaviour.
- Only the GAERS develop a depressive-like behaviour, in contrast to the NEC, GAERS-Cacna1h^{NEC} and NEC-Cacna1h^{GAERS} indicating that the R1584P variant is a determinant of this behaviour.
- T-type Ca^{2+} channel isoform $\text{Ca}_v3.2$ channel expression was increased in GAERS only, and the ratio of $\text{Ca}_v3.2$ +25/-25 splice variants were increased in GAERS and NEC-Cacna1h^{GAERS} compared to NEC and GAERS-Cacna1h^{NEC} indicating a role for the R1584P in determining this ratio.