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Cacna1g is a genetic modifier of epilepsy in a mouse model of Dravet syndrome

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Summary

Dravet syndrome, an early onset epileptic encephalopathy, is most often caused by *de novo* mutation of the neuronal voltage-gated sodium channel gene *SCN1A*. Mouse models with deletion of *Scn1a* recapitulate Dravet syndrome phenotypes, including spontaneous generalized tonic-clonic seizures, susceptibility to seizures induced by elevated body temperature, and elevated risk of sudden unexpected death due to epilepsy. Importantly, the epilepsy phenotype of Dravet mouse models is highly strain-dependent, suggesting a strong influence of genetic modifiers. We previously identified *Cacna1g*, encoding the Cav3.1 subunit of the T-type calcium channel family, as an epilepsy modifier in the $Scn2a^{Q54}$ transgenic epilepsy mouse model. In this study, we asked whether transgenic alteration of *Cacna1g* expression modifies severity of the $Scn1a^{+/-}$ Dravet phenotype. $Scn1a^{+/-}$ mice with decreased *Cacna1g* expression showed partial amelioration of disease phenotypes with improved survival and reduced spontaneous seizure frequency. However, reduced *Cacna1g* expression did not alter susceptibility to hyperthermia-induced seizures. Transgenic elevation of *Cacna1g* expression had no effect on the $Scn1a^{+/-}$ epilepsy phenotype. These results provide support for *Cacna1g* as a genetic modifier in a mouse model of Dravet syndrome and suggest that Cav3.1 may be a potential molecular target for therapeutic intervention in patients.

Keywords

Seizures; Epilepsy; Mouse model; Voltage-gated ion channels; Voltage-gated sodium channels; Voltage-gated calcium channels; Genetics

Introduction

Dravet syndrome is an epileptic encephalopathy most often caused by *de novo* heterozygous loss of function mutation in the neuronal voltage-gated sodium channel gene *SCN1A*.[1] In addition to pleiomorphic seizures that are often refractory to treatment, Dravet syndrome includes co-occurring neurodevelopmental delay and elevated risk of sudden unexpected

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death due to epilepsy (SUDEP).[1] A number of mouse models with heterozygous deletion of *Scn1a* have been developed to better understand the underlying mechanisms of Dravet syndrome and to develop novel treatment strategies.[2–4]

Scn1a$^{+/-}$ mice experience spontaneous generalized tonic-clonic seizures, seizures provoked by elevated body temperature, and reduced lifespan, similar to individuals with Dravet syndrome.[2–4] Interestingly, the epilepsy phenotype of *Scn1a*$^{+/-}$ mice is highly strain-dependent.[2,4] *Scn1a*$^{+/-}$ mice maintained on a 129 strain (either 129S6/SvEvTac or 129/SvJ) are unaffected, with no evidence of spontaneous seizures and a normal lifespan.[2,4] In contrast, when crossed to C57BL6/J (B6), [B6x129]F1.*Scn1a*$^{+/-}$ mice display hallmark Dravet phenotypes including spontaneous seizures, reduced lifespan, and susceptibility to hyperthermia-induced seizures.[2,4] Whole genome mapping identified several genetic modifier loci (*Dsm1-5*) that influence survival of *Scn1a*$^{+/-}$ mice.[4] Fine mapping of the *Dsm1* locus on mouse chromosome 5 resulted in the identification of *Gabra2* as a modifier of the *Scn1a*$^{+/-}$ Dravet mouse model.[5] Another locus was mapped to chromosome 11, although the 1.5-LOD support interval was proximal to *Cacna1g*.[5] These observations support the hypothesis that genetic modifiers influence expressivity of epilepsy in Dravet syndrome.

We previously identified *Cacna1g*, encoding the Cav3.1 subunit of the T-type calcium channel family, as a genetic modifier in the *Scn2a*^{Q54} transgenic epilepsy mouse model.[6,7] Transgenic alteration of *Cacna1g* expression influenced the *Scn2a*^{Q54} phenotype.[7] Other epilepsy modifier genes, including *Hlf* and *Kcnq2*, influence phenotypes in both *Scn2a*^{Q54} and *Scn1a* epilepsy mouse models.[8,9] In the current study, we evaluated the effect of transgenic alteration of *Cacna1g* expression on the *Scn1a*$^{+/-}$ epilepsy phenotype. *Scn1a*$^{+/-}$ mice with decreased *Cacna1g* expression exhibited improved survival and decreased spontaneous seizure frequency. These results provide support for *Cacna1g* as a genetic modifier in a mouse model of Dravet syndrome and suggest that Cav3.1 may be a potential molecular target for therapeutic intervention in patients.

Methods

Mice

Scn1a^{tm1Kea} (1A$^{+/-}$) mice (MMRRC stock #37107-JAX) congenic on 129S6/SvEvTac (129) were previously described and are maintained by continued backcrossing to 129S6/SvEvTac (Taconic).[4] The global heterozygous null *Cacna1g*$^{+/-}$ (B6.1G$^{+/-}$) line was previously described and is maintained by backcrossing of heterozygous B6.1G$^{+/-}$ offspring to C57BL/6J (Jackson Labs 000664).[10] The previously described B6.*Cacna1g*HIGH (B6.1GH) BAC transgenic line Tg(RP23-65I14)1Kea (MMRRC stock #42089-JAX), with elevated *Cacna1g* expression, is maintained by backcrossing of hemizygous mice to C57BL/6J.[7] B6.1G$^{+/-}$ and B6.1GH females were crossed with 129.1A$^{+/-}$ males to generate double transgenic (1A$^{+/-}$;1GH or 1A$^{+/-}$;1G$^{+/-}$) mice and single mutant littermate controls. Mice were genotyped by PCR of DNA isolated from postnatal day 14 (P14) tail biopsies as described.[7]

Mice were group-housed with access to food and water *ad libitum* and maintained on a 14-hour light/10-hour dark schedule. All studies were approved by the Northwestern University

Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Principles outlined in the ARRIVE guideline and Basel declaration were considered when planning the experiments. There were no significant differences between sexes on any measurements so groups were collapsed across sex.

Survival

At P21, mice were weaned into cages containing 4–5 mice of the same sex and survival was monitored to 8 weeks of age. All mice were monitored daily for general health and any mouse visibly unhealthy (e.g. underweight, dehydrated, poorly groomed, or immobile) was euthanized and excluded from the study. Survival cohorts were analyzed using Mantel-Cox logrank and $p < 0.05$ was considered statistically significant.

Hyperthermia-induced seizure threshold

Susceptibility to hyperthermia-induced seizures was tested as previously described.[12] Body temperature was recorded when a generalized tonic-clonic seizure (GTCS) occurred. Hyperthermia-induced seizure threshold was analyzed using Mantel-Cox logrank and $p < 0.05$ was considered statistically significant.

Continuous video monitoring of spontaneous seizure activity

Mice were phenotyped as previously described.[5,12] Prior extensive video-electroencephalogram monitoring of $1A^{+/-}$ mice demonstrated a perfect correlation between behavioral and electroencephalographic seizures ($\kappa = 1.0$), validating visual assessment of GTCS.[5] Behavioral seizures were assessed offline by an observer blinded to genotype, counting the number of GTCSs. A 48-hour window of time from 24:00 on P22 to 24:00 on P24 was reviewed for each subject.

A separate cohort of mice were subjected to a single, brief hyperthermia-induced GTCS event at P18 and then were video-monitored for spontaneous GTCS until P22.[12] A 60-hour window of time from 12:00 on P19 to 24:00 on P21 was reviewed. Seizure frequencies were compared between genotypes using Mann-Whitney ranksum test with $p < 0.05$ being considered statistically significant.

Video-electroencephalogram (video-EEG)

Video-EEG experiments were performed as previously described.[5,8] Briefly, mice were implanted with prefabricated headmounts (Pinnacle Technology, Inc) under isoflurane anesthesia. After 48 hrs recovery, mice underwent continuous video-EEG monitoring until at least P26. EEG and synchronized video recordings were reviewed manually to identify electrographic seizures and behavioral correlates. All electrographic seizures correlated with behavioral tonic-clonic convulsions with loss of posture. A total of five (three males; two females) $1A^{+/-};1G^{+/-}$ mice were evaluated by video-EEG (857 total hours). There was no evidence of electrographic seizures in two $1G^{+/-}$ mice evaluated by video-EEG (381 total hours). Previous extensive video-EEG recoding of Cacna1g BAC transgenic mice revealed spike-wave discharges, but no GTCS.[13]

Transcript analysis

Cacna1g mRNA expression levels were analyzed similarly as described previously.[7] Brains were collected from P19 mice 24 hours after a single hyperthermia-induced GTCS event or sham treatment (10 minute hold at 37 °C). Total RNA was extracted from whole brains using Trizol (Life Technologies) and first-strand cDNA was generated using Super-Script III (Life Technologies). Quantitative droplet digital PCR (ddPCR) was performed using ddPCR Supermix for Probes (No dUTP) (Bio-Rad) and TaqMan Gene Expression Assays (Life Technologies) for *Cacna1g* (FAM_Mm00486571_m1) and TATA binding protein (*Tbp*) (VIC_Mm00446971_m1). Reactions were partitioned into droplets, amplified, and analyzed according to manufacturer's recommendations (Bio-Rad). Relative transcript levels were expressed as a ratio of *Cacna1g*/*Tbp* concentration and compared between groups by Student's t-test with $p < 0.05$ being considered statistically significant.

RNA-seq

Sequence-based transcriptome analysis of 129S6/SvEvTac and [C57BL/6 × 129S6/SvEvTac]F1 hippocampus was previously reported.[5] Briefly, sequence reads were performed on the Illumina HiSeq4000 platform, processed with the Illumina pipeline, and analyzed with a Tophat-htseq-Deseq2 pipeline on the GALAXY open, web-based platform.

Results

To determine whether altered expression of *Cacna1g* would affect the epilepsy phenotype of heterozygous null *Scn1a*$^{+/-}$ mice, B6.1G$^{+/-}$ mice were crossed with 129.1A$^{+/-}$ mice to generate single and double mutant mice on an F1[B6x129] background. Single and double mutant mice were monitored for survival to eight weeks of age. We observed improved survival with reduced *Cacna1g* expression in 1A$^{+/-}$;1G$^{+/-}$ mice relative to single mutant 1A$^{+/-}$, while 1G$^{+/-}$ littermates exhibited 100% survival (Fig. 1A). The observed improvement in survival correlated with a reduction in spontaneous seizure frequency. Video-monitoring of spontaneous seizures during the period of highest seizure frequency (P23-24) revealed a significant reduction in spontaneous seizure frequency in 1A$^{+/-}$;1G$^{+/-}$ mice relative to 1A$^{+/-}$ littermates (Fig. 1B). Video-EEG analysis confirmed that reduction in behavioral seizures observed in mice with reduced *Cacna1g* expression correlated with a reduction in electrographic seizures. Furthermore, electrographic seizures that were observed in a single male 1A$^{+/-}$;1G$^{+/-}$ mouse had behavioral correlates indistinguishable from seizures in 1A$^{+/-}$ mice. The remaining 1A$^{+/-}$;1G$^{+/-}$ mice, as well as single mutant 1G$^{+/-}$ mice, had no evidence of electrographic seizures.

We recently reported that inducing a single, brief hyperthermia-priming GTCS in P18 F1.1A$^{+/-}$ mice results in a robust increase in the incidence of subsequent spontaneous GTCSs for at least three days.[12] To determine whether reduced *Cacna1g* expression influences spontaneous seizures following hyperthermia-priming, mice were monitored by continuous video recording for 60 hours. The temperature threshold for the hyperthermia-induced priming seizure was similar among 1A$^{+/-}$ and 1A$^{+/-}$;1G$^{+/-}$ mice (mean seizure threshold of 42.05 ± 0.33°C for 1A$^{+/-}$ mice and 42 ± 0.32°C for 1A$^{+/-}$;1G$^{+/-}$ mice; n=22 per group for both groups; $p_{Logrank}=0.689$). Surprisingly, there was no significant difference in

spontaneous GTCS frequency between $1A^{+/-}$ and $1A^{+/-}$;$1G^{+/-}$ mice following hyperthermia-priming (Fig. 1C).

To examine the paradoxical effects of reduced *Cacna1g* expression on spontaneous GTCS frequency in naïve versus hyperthermia-primed mice, we assessed whether *Cacna1g* expression was altered by seizure activity in the $Scn1a^{+/-}$ Dravet model. At P18 mice were subjected to a single, brief hyperthermia-induced GTCS or sham treatment. Whole brain RNA was collected 24 hours post treatment. We observed increased *Cacna1g* mRNA levels after a hyperthermia-priming seizure relative to mice in the sham treatment group (Fig. 2A). In an independent set of $1A^{+/-}$ mice, hippocampi were collected at P24 following video monitoring of spontaneous seizure frequency without prior hyperthermia-priming. *Cacna1g* mRNA levels were elevated in samples from mice exhibiting spontaneous GTCSs in the preceding 24 hours relative to seizure-free littermates (Fig. 2B; FDR-adjusted $p=2.57e^{-28}$ (Benjamini Hockberg)). Elevation of *Cacna1g* mRNA levels post-priming may counteract the protective modifier effect of the heterozygous *Cacna1g* null allele in the $Scn1a^{+/-}$ model. Interestingly, transgenic elevation of *Cacna1g* expression did not affect spontaneous GTCS frequency (mean seizure frequency of 1.69 ± 0.92 seizures/day for $1A^{+/-}$ mice and 1.15 ± 0.75 seizures/day for $1A^{+/-}$;1GH; n=7–8 per group; $p_{Mann\text{-}Whitney}=0.50$) or survival (median survival of 34.5 days for $1A^{+/-}$;1GH and 25 days for $1A^{+/-}$ mice, n=29–40 per group, $p_{Logrank}=0.175$; 56 days for 1GH mice, n=15) in the $Scn1a^{+/-}$ Dravet model, suggesting the possibility that modest upregulation of *Cacna1g* expression post-seizure is not a proximate cause of GTCS.

Discussion

We previously demonstrated *Cacna1g* acts as a modifier of epilepsy in the $Scn2a^{Q54}$ model of focal epilepsy.[7] Additionally, another modifier gene, *Hlf*, was found to affect both the $Scn1a^{+/-}$ and $Scn2a^{Q54}$ epilepsy models.[9] While both are caused by mutation or deletion of a major central nervous system voltage-gated sodium channel, the epilepsy phenotypes and underlying mechanisms of these two models are quite distinct. The $Scn2a^{Q54}$ model is characterized by a progressive phenotype that begins with frequent focal motor seizures and then progresses to include GTCSs and premature death.[14] The $Scn1a^{+/-}$ model recapitulates hallmark Dravet phenotypes and exhibits spontaneous GTCSs, reduced lifespan, and susceptibility to hyperthermia-induced seizures, thought to be a correlate of thermally-provoked seizures in Dravet syndrome.[2–4] Interestingly, the phenotypes of both the $Scn1a^{+/-}$ and $Scn2a^{Q54}$ epilepsy models are highly strain dependent.[2,4,15] Our observations that at least two independently identified modifier genes (*Hlf* and *Cacna1g*) influence the phenotypes in both the $Scn1a^{+/-}$ and $Scn2a^{Q54}$ models suggest that common modifier pathways are able to modify epilepsies with distinct epileptogenic mechanisms.

While ethosuximide, a T-type calcium channel inhibitor, is utilized for the management of atypical absence seizures in Dravet syndrome, the potential for therapeutic benefit for other seizure types is unclear. Future preclinical evaluation of T-type calcium channel blockers like ethosuximide in $Scn1a^{+/-}$ mice will help determine the utility of pharmacologic blockade of these channels for the treatment of Dravet syndrome.

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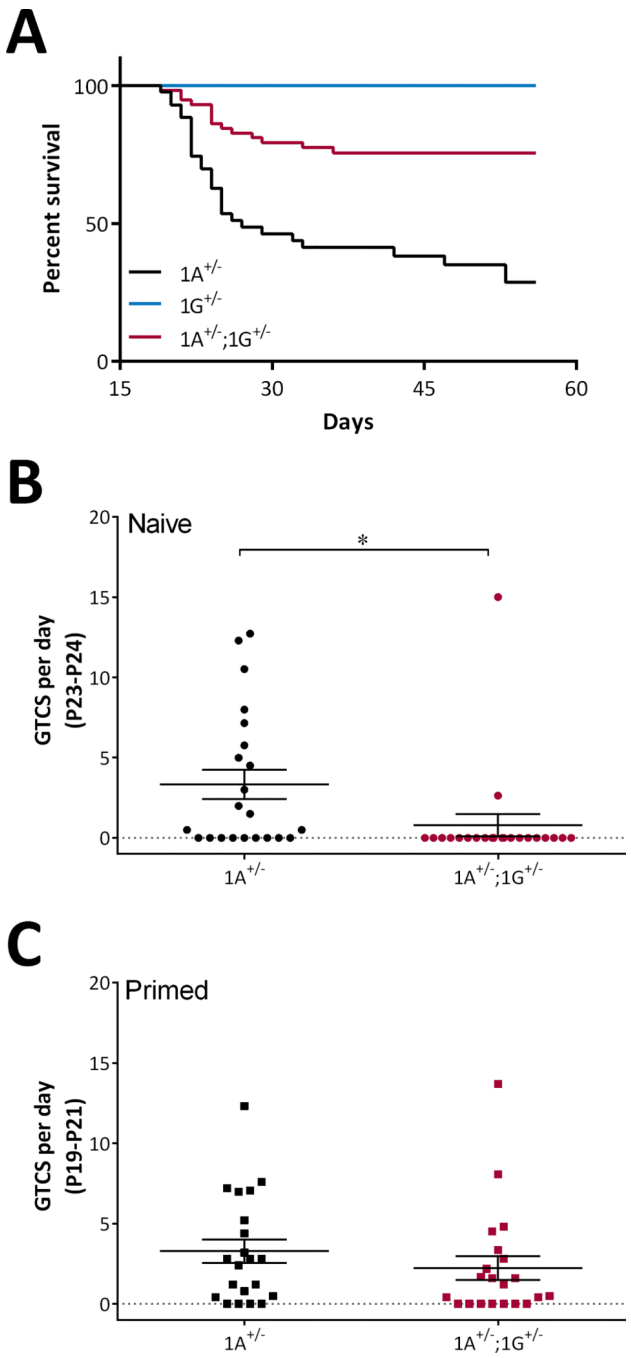

Figure 1. Effect of reduced *Cacna1g* expression on *Scn1a*$^{+/-}$ survival and spontaneous seizure rate

(A) Survival over 8 weeks is shown for control 1A$^{+/-}$ (black), 1G$^{+/-}$ (blue) mice and 1A$^{+/-}$;1G$^{+/-}$ (red) mice. Reduced expression of *Cacna1g* resulted in a significant improvement in survival of 1A$^{+/-}$;1G$^{+/-}$ versus 1A$^{+/-}$ mice. $p < 0.0001$ (Mantel-Cox Logrank), with n=43–58 per group. (B) Spontaneous seizure rate during a 48 hour window is displayed. Fewer seizures per hour were observed in 1A$^{+/-}$;1G$^{+/-}$ mice (red circles) relative to control 1A$^{+/-}$ (black circles) littermates. *$p < 0.0009$ (Mann-Whitney U test). Average seizure frequencies are depicted by the horizontal line and error bars represent standard error of the mean

(SEM), with n = 22 per group. (C) Spontaneous seizure rate during a 60 hour window following a hyperthermia-induced priming seizure is displayed. No difference in seizures per hour were observed in $1A^{+/-};1G^{+/-}$ mice (red squares) relative to control $1A^{+/-}$ (black squares) littermates. $p > 0.05$ for both males and females (Mann-Whitney U test). Average seizure frequencies are depicted by the horizontal line ± SEM, with n = 21 per group.

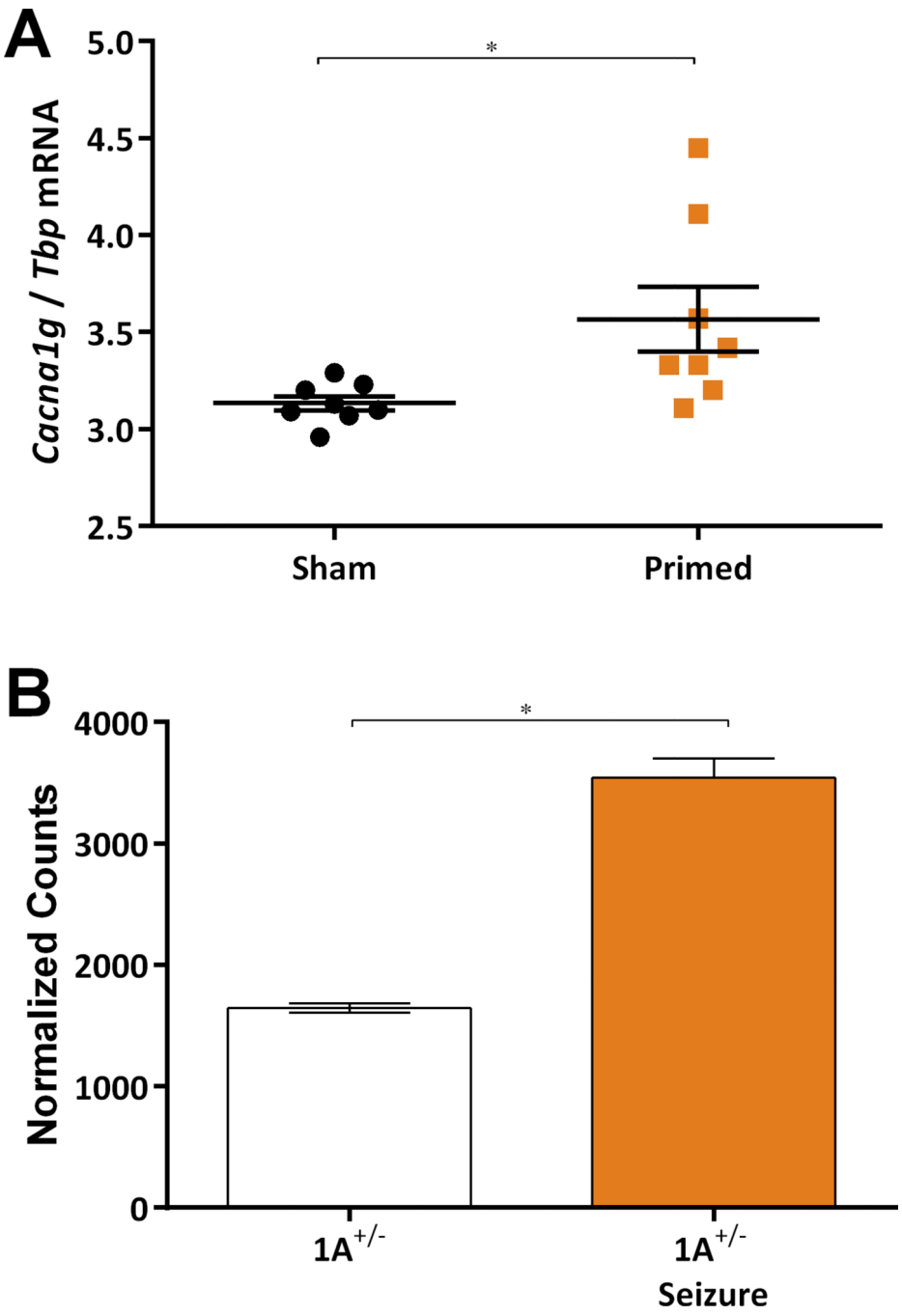

Figure 2. *Cacna1g* mRNA levels increase after seizure activity

(A) To assess expression at the level of messenger RNA (mRNA), a quantitative droplet digital PCR (ddPCR) Taqman assay was performed on whole brain samples collected from P19 $1A^{+/-}$ mice 24 hours after either a priming event (hyperthermia-induced GTCS) or a sham treatment. The expression of *Cacna1g* was normalized to *Tbp* expression. p-value was determined by unpaired Student's t-test with Welch's correction. Error bars represent upper and lower limits derived from standard error of the mean. Mean *Cacna1g* expression was observed to be the following: Sham = 3.13 (n = 8) and Primed = 3.56 (n = 8); *p < 0.05. (B)

Differences in hippocampal *Cacna1g* expression between $1A^{+/-}$ mice with and without observed seizure activity prior to sample collection. RNA-seq normalized count values are shown. *FDR adjusted p-value = $2.57e^{-28}$ (Benjamini-Hochberg).